LACTATE AS A MAJOR SIGNALING MOLECULE FOR HOMEOSTASIS

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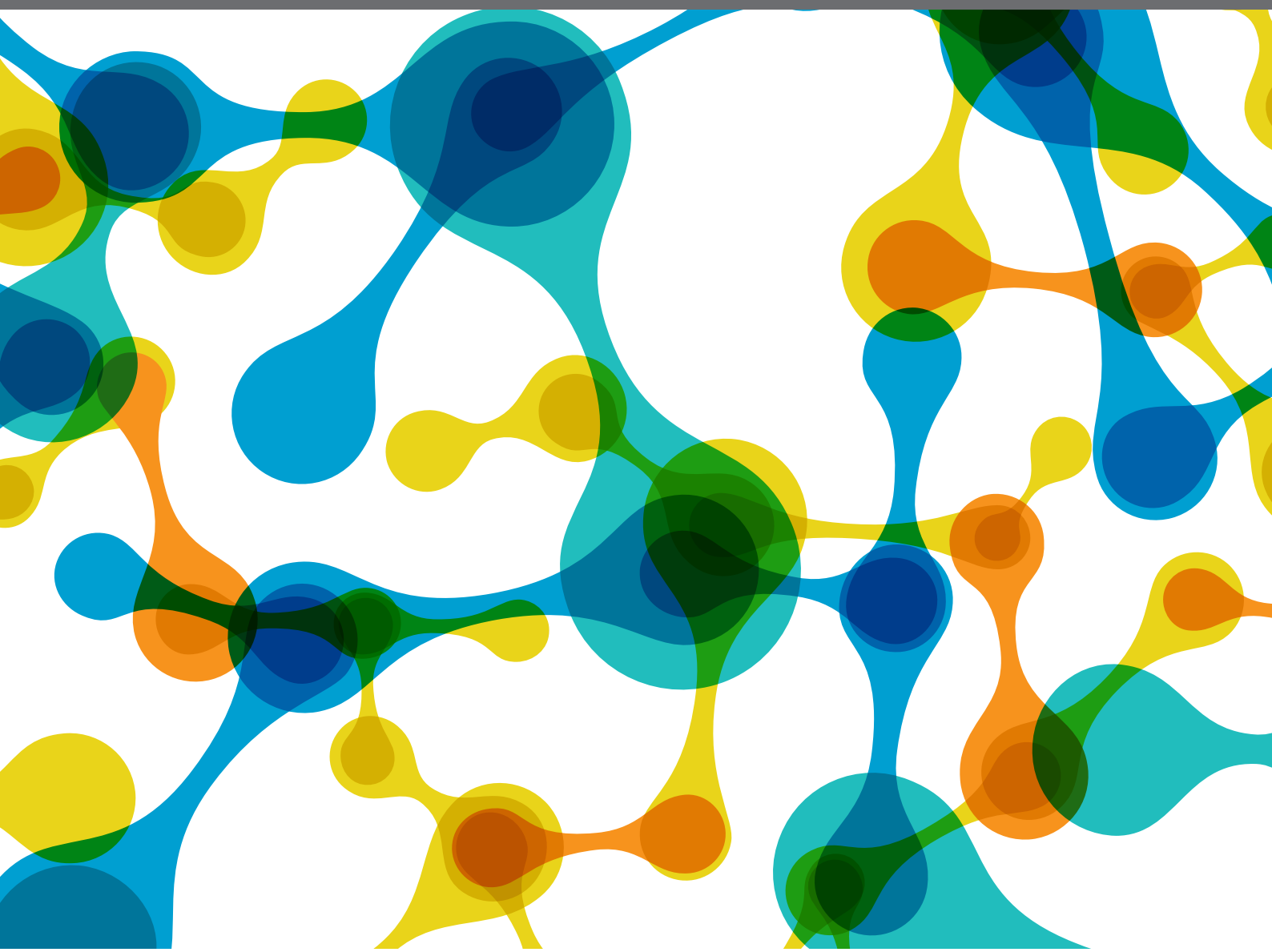

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LACTATE AS A MAJOR SIGNALING MOLECULE FOR HOMEOSTASIS

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EDITORIAL
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Editorial: Lactate as a Major Signaling Molecule for Homeostasis

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Editorial on the Research Topic

Lactate as a Major Signaling Molecule for Homeostasis

L-lactate metabolism was first investigated in exercise physiology since it was considered as a waste product of glycolysis due to oxygen deficiency. Since the 1980s', several studies showed that lactate is an essential metabolic fuel and signaling molecule (Brooks, 2020). Then, the interest for lactate has extended to a growing number of disciplines, from physiology to pathology and exercise is now viewed as a metabolic trigger for tissues adaptation.

Lactate is transported by the monocarboxylate transporters (MCTs) family which present a ubiquitous tissue distribution but lactate can additionally activate the hydroxycarboxylic acid receptor 1 (HCAR1) also known as G protein-coupled receptor 81 (GPR81). In most tissues and cell types, lactate entry is mediated by MCT1 or MCT2 and related to oxidative capacity whereas MCT4 would be mainly involved in lactate output. The basic knowledge on lactate is that it could be a metabolic substrate or exchange molecule for muscle (Brooks), or brain (Pellerin, 2010), that MCTs content vary with training (Dubouchaud et al., 2000), nutritional status (Lambert et al., 2003; Pierre et al., 2007), cellular activity or intracellular signaling (Pérez-Escuredo et al., 2016) and diseases (Py et al., 2001).

This Research Topic provides an update about the knowledge of lactate roles and mechanisms of action. Durand et al., focused on blood lactate kinetics in response to exercise. Their findings showed that the longer the recovery period, the better is the quality of models to describe lactate exchange and removal abilities. In the brain, exercise allows to increase lactate release, favoring hippocampal metabolism and especially mitochondrial biogenesis (Park et al.) but also mitochondrial efficiency and brain-derived neurotrophic factor biosynthesis (Hu et al.) (**Figure 1**). The ability of lactate to increase mitochondrial biogenesis has been previously described in skeletal muscle. The present studies demonstrated that lactate and oxidative metabolism could play an important role in the physiology of other tissues. Indeed, the effects of exercise on hippocampal mitochondria number and function highlight the role of lactate in mediating memory processes and, in turn, physical performance. Lactate is a mediator of neuron-astrocyte dialogue since astrocytes provide lactate to neuron as an energetic substrate. Horvat et al. have provided evidence of this relationship with a focus on the lactate-positive feedback mechanism in astrocytes. Astrocytes switch their metabolism to lipid metabolism, enhancing availability of lactate for neuron as a metabolic substrate when ATP needs to be increased. The control and regulation of lactate entry into astrocytes could involve known and unknown transporters. Interestingly, during stressful conditions, such as hypoxia or ischemia-reperfusion (I/R), increasing lactate concentration *via* exogenous lactate perfusion ameliorates the neurological outcome (Roumes et al., 2021). Buscemi et al. have tested the hypothesis that the beneficial effect of lactate in cerebral I/R process could be due to the activation of HCAR1 by

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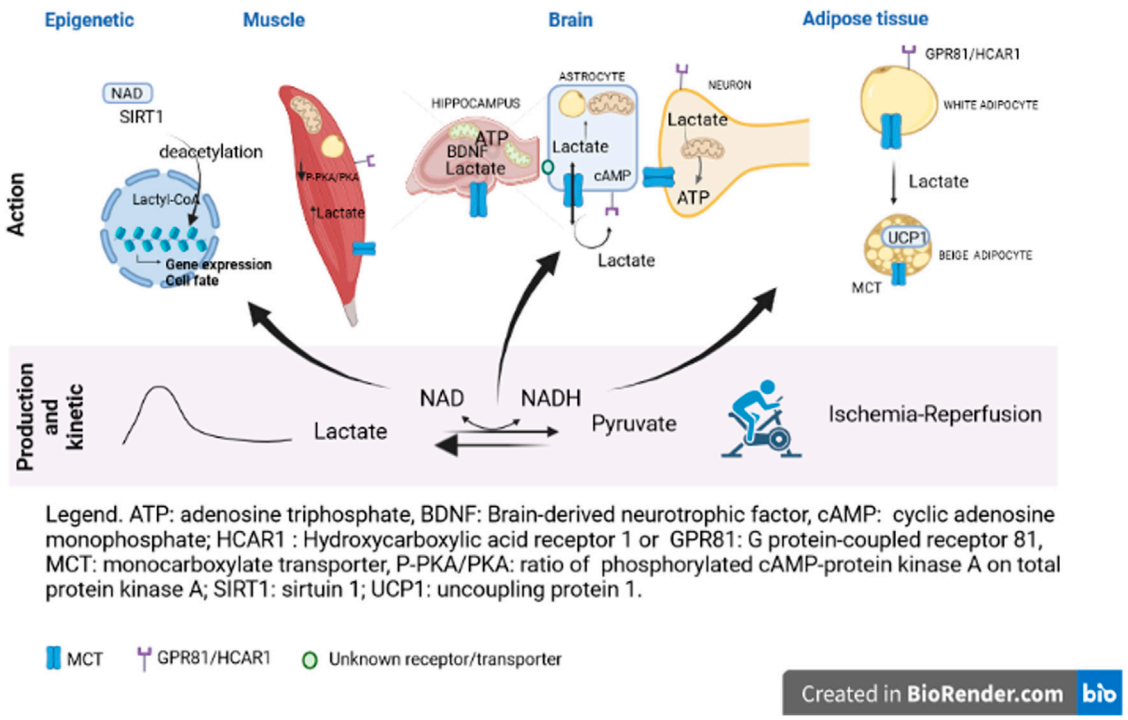

FIGURE 1 | ATP, adenosine triphosphate; BDNF, brain-derived neutrophic factor; cAmp, cyclic adenosine monophosphate; HCAR1, hydroxycarboxylic acid receptor 1 or GPR81, G protein-coupled receptor 81; MCT, monocarboxylate transporter; P-PKA/PKA, ratio of phosphorylated cAMP-protein kinase A on total protein kinase A; SIRT1, sirtuin 1; UCP1, uncoupling protein 1.

lactate. However, HCAR1 agonists did not exert a protective effect neither on lesion size nor on neurological outcome. A role of other transporter(s)/receptor(s) or metabolite(s) in this process is suggested.

The signaling pathway(s) activated by lactate are still unclear. Chen et al. provided interesting results suggesting the implication of the cAMP-PKA pathway. Chronic lactate injection, mimicking lactate variation during exercise, induced intramuscular triglycerides accumulation mainly through inhibition of lipolysis *via* a decrease of PKA activation. However, citrate synthase protein level and lactate concentration were still high even after PKA activation by forskolin. These findings suggest that lactate could regulate oxidative capacity through mitochondrial metabolite derivatives. This relationship could participate to the increase in performance of athletes but would be lost in muscle of obese patients. In metabolic diseases, hyperlactatemia is not associated with increased oxidative capacity, underlining a dysregulation of lactate signaling.

Adipose tissues, like skeletal muscles, have a remarkable cell and metabolic plasticity and a large ability to adapt their phenotype in response to their environment. The review of Lagarde et al. discussed the adaptability of white, brown and beige adipocytes. The authors illustrated how adipocytes are especially adapted to control the redox equilibrium at cellular and whole-body level. The adipose tissues responses to lactate exposure could be part of the tissue stress adaptation. In pathological context, an alteration of adipose tissues lactate response would disturb whole-body redox state level favoring an impairment of redox homeostasis and whole-body metabolism.

Beside its role in redox homeostasis, lactate is a strong candidate as a promoter of different cell fates such as for satellite cells and immune cells. Lactate is a regulator of redox potential *via* the NAD/NADH *ratio* leading to sirtuin deacetylases, like SIRT1, activation that will alter histones acetylation and gene expression. The epigenetic role of lactate is not solely related to its metabolic activity but also to a new process called lactylation (Zhang et al., 2019). Although there is no direct clues of satellite cell activation by lactate, the review of Nalbandian et al. discussed different experiments suggesting a role of lactate in muscle regeneration *via* activation and proliferation of satellite cells.

Several immune cell type profiles are shaped by lactate and its associated proton leading to modification of their function. As mentioned in the review of Caslin et al., immune cell metabolism has gained attention only recently. The inhibition of glycolysis in inflammatory immune cells by lactate would promote anti-inflammatory and immunosuppressive processes. However, depending on the environment, epigenetic alterations, acute or chronic inflammation, immune cells in the course of a pathological process will exhibit a different response to lactate exposure.

Although lactate has been discovered more than two centuries ago, there is still a need for research to understand lactate actions in physiology and diseases. How lactate coordinates redox metabolism at cell, tissues and whole-body level? How lactate crosses cellular membranes with a regulation at transporter/receptor levels? How lactate and glycolysis are involved in epigenetic alterations? What signaling pathways are activated by lactate? Thus, although lactate is recognized today as a cornerstone of tissues adaptation and communication, future studies are still needed to further clarify its numerous roles.

Finally, the editors would like to thank all contributors and reviewers that allowed this Research Topic to become a success in drawing attention to a long-neglected (but so interesting!) metabolite.

AUTHOR CONTRIBUTIONS

KL wrote the first version of the Editorial and CB, PC and LP carefully reviewed it. All authors approved the final version.

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MINI REVIEW
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Lactate Metabolism and Satellite Cell Fate

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Lactate is one of the metabolic products of glycolysis. It is widely accepted as an important energy source for many cell types and more recently has been proposed to actively participate in cell-cell communication. Satellite cells (SCs), which are adult skeletal muscle stem cells, are the main players of the skeletal muscle regeneration process. Recent studies have proposed a metabolic switch to increase glycolysis in activated SCs. Moreover, lactate has been shown to affect SCs and myoblasts *in vivo* and *in vitro*. In this short review, we describe how metabolic variations relate with SC fate (quiescence, activation, proliferation, migration, differentiation, fusion, and self-renewal), as well as discuss possible relationships between lactate as a metabolite and as a signaling molecule affecting SC fate.

Keywords: lactate, muscle stem cell, metabolism, skeletal muscle, muscle regeneration

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INTRODUCTION

Satellite cells (SCs) are the main players responsible for the regeneration and maintenance of human adult skeletal muscle (Chargé and Rudnicki, 2004). During homeostasis, SCs are in a quiescent state with relatively low metabolic demands (Rodgers et al., 2014; Pala et al., 2018). Under stress, however, caused by injury, exercise, or other variables, SCs become activated to proliferate, differentiate, and fuse to regenerate the tissue. The transcriptional cascade that leads to these events has been deeply studied (Baghdadi and Tajbakhsh, 2018), but the mechanism that leads to these transcriptomic activations is not fully understood. Recent research has suggested that external factors such as metabolites or factors released by surrounding cells cause the activation of SCs (Tsuchiya et al., 2020; Zhang et al., 2020).

Interestingly, glycolysis has been related to many mechanisms that regulate epigenetic modifications in SCs (Ryall et al., 2015; Yucel et al., 2019). Lactate, which is produced by glycolysis, is easily transported between cells, making it a candidate mediator for these glycolysis effects. Furthermore, one recent study reported a new epigenetic modification process called lactylation, which is regulated by lactate (Zhang et al., 2019). However, little research has given attention to the possibility that lactate may regulate the epigenetic modifications that determine the SC state. In this short review, we summarize recent findings regarding SC metabolism and its effect on SC fate, with special consideration on lactate.

ROLE OF SATELLITE CELLS IN SKELETAL MUSCLE

Healthy skeletal muscle is characterized by a high regenerative capacity (Chargé and Rudnicki, 2004). This regenerative capacity is attributed to the presence of SCs (Mauro, 1961). SCs are located beneath the basal lamina and close to the capillaries (Christov et al., 2007; Nederveen et al., 2016; Verma et al., 2018). Proper SC function is critical for not only the regeneration of adult skeletal muscle, but also its maintenance including muscle mass (Bentzinger et al., 2012).

During homeostasis, SCs exist in a quiescent state. Upon injury, SCs enter the cell cycle to become activated and undergo several rounds of proliferation, a process that happens very quickly from the 1st days after injury (Baghdadi et al., 2018). During proliferation, asymmetric and symmetric divisions happen. Symmetrically divided cells (mostly planar divisions) can give rise to two activated SCs or two SCs committed to myogenesis, while asymmetrically divided cells (mostly apical-basal division) will give rise to one activated SC and one quiescent SC, which contributes to the self-renewal of SCs (Cossu and Tajbakhsh, 2007; Kuang et al., 2007; Motohashi and Asakura, 2014). Activated SCs possess increased migratory capacity, which allows them to migrate to the injured sites (Baghdadi et al., 2018; Evano and Tajbakhsh, 2018). Once they reach there, the majority of activated SCs will start to differentiate into myoblasts and fuse to each other and to existing fibers to regenerate the injured site. A smaller population of active SCs will re-enter the quiescent state to maintain the SC pool (Rocheteau et al., 2012).

Satellite cells have been deeply studied, and the transcriptional program that regulates their fate has been established (Baghdadi and Tajbakhsh, 2018). Quiescent SCs are characterized by the expression of PAX7 (Seale et al., 2000), a paired-type homeobox transcription factor that is recognized as the major marker of adult SCs. After activation, PAX7 expression is decreased, and myogenic regulatory factors (MRFs) are expressed. MRFs are a family of basic helix-loop-helix (bHLH) transcription factors composed of MYF5, MYOD, MRF4, and MYOGENIN that promote the expressions of skeletal muscle-specific genes, determining which cells commit to myogenic differentiation (Pownall et al., 2002; Zammit, 2017; Asfour et al., 2018). When SCs become activated, MYF5 and MYOD expression is increased, while PAX7 expression decreases such that its protein level is undetectable in proliferating SCs. Later, cells that undergo differentiation will start expressing MYOG and MRF4 to exit the cell cycle and fully commit into myogenic differentiation (Baghdadi and Tajbakhsh, 2018).

Besides the transcription factors that determine SC fate, epigenetic changes in SCs have been shown to regulate myogenic differentiation (Liu et al., 2013; Wang et al., 2019; Shcherbina et al., 2020). Histones are proteins that provide structural support for packing DNA. Many histone modifications are known to be important for regulating transcriptional activity (Strahl and Allis, 2000). Consistently, when transitioning from the quiescent to activated state, SCs undergo several histone modifications associated with transcriptional changes that lead to myogenic differentiation (Liu et al., 2013). Furthermore, growing evidence has linked histone modifications with metabolism (Sabari et al., 2017).

METABOLIC REGULATION OF SATELLITE CELLS DURING REGENERATION

Different studies have shown that the metabolic profiles differ between quiescent, activated, and differentiated SCs, indicating that metabolic changes may affect SC fate (Ryall, 2013; Tang and Rando, 2014; Ryall et al., 2015; Pala et al., 2018; Yucel et al., 2019).

The majority of adult stem cells exist in a quiescent state and have low metabolic demand (Ito and Suda, 2014). SCs have no exception to this rule. It has been reported that after injury, SCs exit quiescence and increase their energy expenditure (Rodgers et al., 2014). Quiescent SCs have a low metabolism characterized by an increase in the expression of genes related to fatty acids oxidation (Fukada et al., 2007; Ryall et al., 2015), suggesting that lipids are the main source of energy for these cells. An RNA-sequencing (RNA-seq) analysis of freshly isolated mouse SCs (quiescent state) and cultured SCs (proliferating state) revealed that the expression of lipid metabolism-related genes is higher in the quiescent SCs than proliferating SCs (Fukada et al., 2007). Similarly, two recent studies (Ryall et al., 2015; Yucel et al., 2019) found that SCs when activated undergo a metabolic shift from lipids to glucose oxidation. When SCs exit the quiescent state, their energetic demands increase (Rocheteau et al., 2012; Rodgers et al., 2014; Dell'Orso et al., 2019; Purohit and Dhawan, 2019). Furthermore, it has been shown that quiescent SCs possess lower levels of mitochondrial activity than activated SCs (Rocheteau et al., 2012; Rodgers et al., 2014). Importantly, one single cell RNA-seq study of SCs in a skeletal muscle mouse injury model confirmed the increased expression of genes related to glycolysis and the tricarboxylic acid (TCA) cycle in activated SCs (Dell'Orso et al., 2019), suggesting that the higher energetic need of the activated SCs is fulfilled by aerobic and anaerobic pathways.

These metabolic changes have been proposed to play important roles in SCs fate. For instance, one study (Theret et al., 2017) suggested that AMP-activated protein kinase (AMPK), which is a master regulator of metabolic homeostasis (Hardie et al., 2012), regulates SC self-renewal by controlling metabolic homeostasis. In that study, AMPK knockout mouse SCs showed a high self-renewal rate and a switch of their metabolism to higher glycolysis, which impaired skeletal muscle regeneration. These findings provided evidence that metabolism and SC fate are connected. At the same time, AMPK has been shown to be regulated by nutritional strategies such as caloric restriction (Canto and Auwerx, 2011). In fact, caloric restriction has been shown to increase SC activity and to enhance skeletal muscle regeneration in mice (Cerletti et al., 2012), strengthening the hypothesis that metabolism regulates SCs fate.

Metabolic changes associated with different SC states have been also correlated with chromatin modifications (Purohit and Dhawan, 2019). Histone acetylation, which is a chromatin modification associated with open chromatin and gene expressions, is regulated in part by the availability of acetyl-CoA (Wellen et al., 2009; Moussaieff et al., 2015). Furthermore, glucose is one of the main precursors for acetyl-CoA; during glycolysis, glucose produces pyruvate, which can be later converted into acetyl-CoA in mitochondria in a process mediated by pyruvate dehydrogenase (PDH). Thereafter, acetyl-CoA is converted to citrate, which can enter the TCA cycle and can be used to produce energy. Moreover, citrate is also transported out of mitochondria and diffuses to nuclei, where it can be used as a substrate to produce acetyl-CoA for histone acetylation. Interestingly, in proliferating SCs, an increase in glucose-derived citrate was found (Yucel et al., 2019). The same study found that this excess of citrate led to an increase in histone acetylation, further leading to a more accessible chromatin that ultimately modulated myogenic differentiation (**Figure 1A**).

Another mechanism has been proposed for the metabolically induced chromatin modifications in activated SCs (Ryall et al., 2015). Metabolic changes can regulate the NAD+-SIRT1 pathway; SIRT1 is a histone deacetylase that uses NAD+ as an enzymatic cofactor to accomplish histone deacetylation (Guarente, 2011; Jing and Lin, 2015). This mechanism, which links metabolic and stress changes with epigenetic modifications of the DNA, is of special relevance to maintaining genomic integrity (Bosch-Presegue and Vaquero, 2015). This mechanism has been suggested to be fundamental for regulating epigenetic processes in stem cells (Fang et al., 2019). Ryall et al. (2015) showed *in vitro* by using cultured mouse SCs and *in vivo* by using SIRT1 deacetylase domain ablation mouse models that the shift from fatty acid oxidation to glycolytic metabolism in activated SCs produces less intracellular NAD+ and consequently less SIRT1 activity, which ultimately leads to histone acetylation and the activation of muscle-specific gene transcription that promotes myogenesis (**Figure 1B**).

Given that lactate is a precursor of pyruvate and its role in reactions that involve NAD+/NADH conversion (see **Figure 2**), and also considering that the NAD+/NADH balance is critical for SIRT1 activation, it is reasonable to think that lactate may have some role in the metabolic control of SC fate with SIRT1 inactivation, favoring SCs activation. Indeed, some studies have suggested that lactate may affect SC commitment by inducing myogenic differentiation (Willkomm et al., 2014; Oishi et al., 2015; Ohno et al., 2019), but no study has reported a direct mechanism yet.

LACTATE AS A KEY METABOLITE IN THE CONTROL OF CELL SIGNALING

Lactate is a metabolite produced from pyruvate by lactate dehydrogenase (LDH), with the LDH isoform A (LDHA) facilitating the pyruvate-to-lactate conversion in cells with high glycolytic rates, and the LDH isoform B (LDHB) facilitating the lactate-to-pyruvate conversion in highly oxidative cells. When the cytoplasmic lactate concentration is elevated, lactate can be co-transported

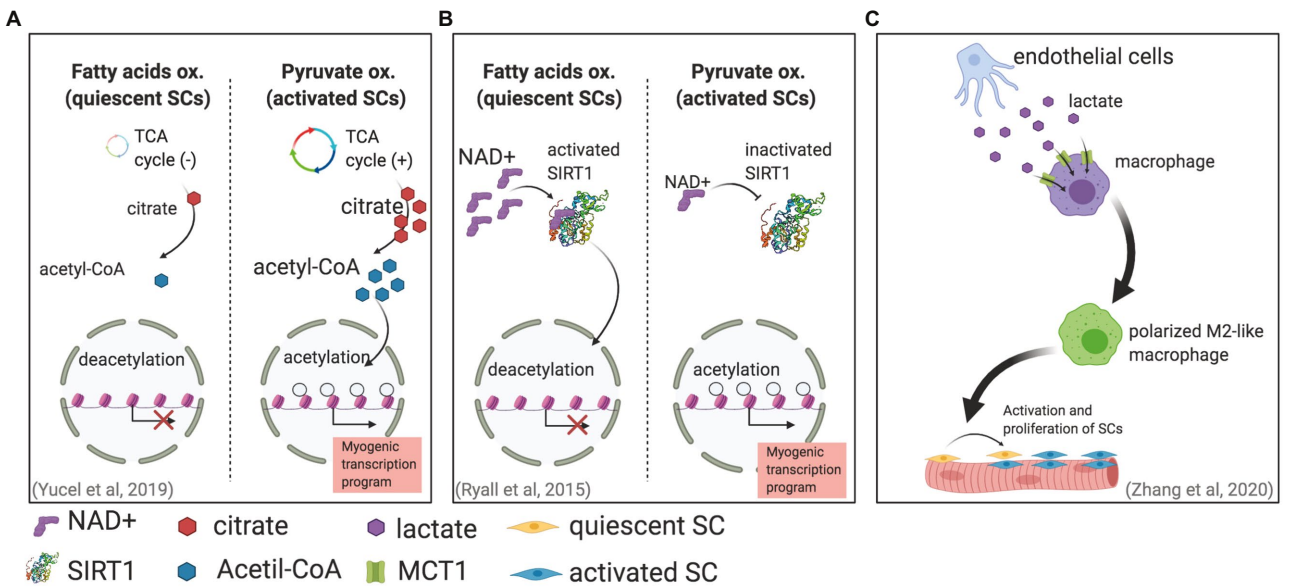

FIGURE 1 | Different mechanisms in which metabolism can affect satellite cell (SC) fate. **(A)** SC activation is associated with an increase in pyruvate oxidation. This increase generates more citrate production, leading to more acetyl-CoA for histone acetylation that will enable gene transcription (Yucel et al., 2019). **(B)** Activated SCs increase pyruvate oxidation, reducing NAD+ and therefore SIRT1 activity. The result is higher histone acetylation and an activated myogenic transcriptional program. **(C)** Endothelial cells can secrete lactate that enters and induces the polarization of macrophages, supporting muscle regeneration by promoting SC proliferation and fusion (Zhang et al., 2020).

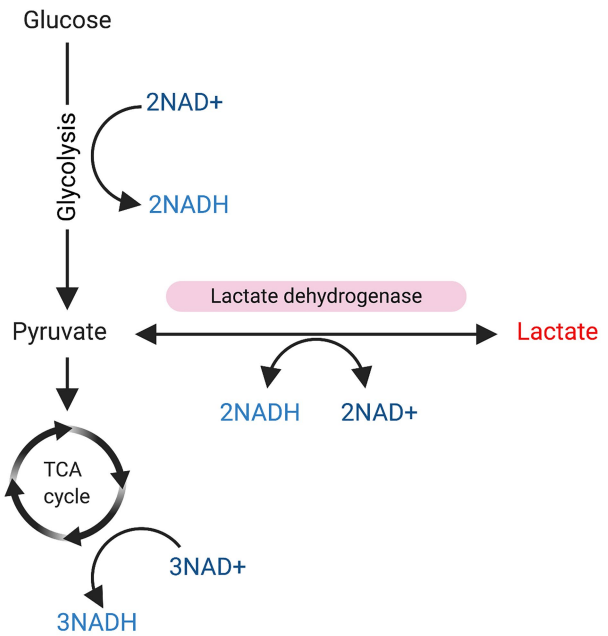

FIGURE 2 | Glycolysis and sources of NAD/NADH+. Glucose is used as a substrate for glycolysis. During glycolysis 2 NAD+ will be converted to NADH, and pyruvate will be produced. Pyruvate can be used as substrate for the tricarboxylic acid (TCA) cycle, resulting in the conversion of 3NAD+ into NADH. On the other hand, pyruvate can be also converted into lactate in a reaction mediated by lactate dehydrogenase, which converts 2NADH to NAD+.

with one H+ ion outside the cell by facilitated diffusion *via* monocarboxylate transporters (MCTs; Halestrap and Wilson, 2012; Kitaoka et al., 2012; Halestrap, 2013; Perez-Escuredo et al., 2016). MCT1 and MCT4 are MCT isoforms expressed in skeletal muscle (Bonen, 2001). MCT1, which has a relatively low Km (3.5–10mM; Halestrap, 2012), is the predominant isoform in oxidative skeletal muscle fibers and considered responsible for lactate uptake (McCullagh et al., 1997; Juel and Halestrap, 1999; Pilegaard et al., 1999; Halestrap, 2012; Chatel et al., 2017). On the other hand, MCT4, which has a much higher Km (22–28mM; Halestrap, 2012), is the isoform predominantly expressed in glycolytic skeletal muscle fibers and considered responsible for lactate release (Dimmer et al., 2000; Fox et al., 2000; Bisetto et al., 2019). Extracellular lactate can travel through the blood stream to many cells, serving as an important energy source for several tissues and organs such as the brain (van Hall et al., 2009; Mosienko et al., 2015), liver, and skeletal muscle (Hui et al., 2017; Brooks, 2020). Given lactate's ability to travel between cells, tissues, and organs, recently it was proposed to be a signaling molecule (Nalbandian and Takeda, 2016; Brooks, 2020).

The idea of lactate being a signaling molecule has been tested in several models. In differentiated C2C12 cells (mouse immortalized myoblasts), culture with L-lactate increased the expression of PGC1-alpha (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), which is a transcription factor responsible for mitochondrial biogenesis (Nalbandian et al., 2019). Similarly, in differentiated L6 cells (immortalized rat myoblasts), culture with lactate increased the expression of PGC1-alpha as well as the expression of several genes related to metabolism like MCT1 (Hashimoto et al., 2007). Moreover, these results were confirmed *in vivo* by the intraperitoneal injection of lactate in mice (Kitaoka et al., 2016), where lactate could increase the skeletal muscle PGC1-alpha expression after 3 h. Additionally, in cancer cells (De Saedeleer et al., 2012; Colegio et al., 2014) and endothelial cells (Sonveaux et al., 2012), lactate induces the activation of hypoxia inducible factor-1 (HIF-1), which is a transcription factor recognized to have an important role in hypoxia and metabolism. Interestingly, HIF-1 has been shown to facilitate lactate transport in cancer cells by upregulating the expression of MCT4 (Ullah et al., 2006).

Together, the above studies indicate that on top of being an energy source, lactate can trigger several tissue-specific mechanisms that lead to the activation of diverse signaling pathways. Because of their location near blood vessels and capillaries (Christov et al., 2007; Verma et al., 2018), SCs may be exposed to high concentrations of lactate. Therefore, in the next section, we will discuss current evidence showing lactate affects SC fate.

POSSIBLE LACTATE METABOLISM EFFECTS ON SATELLITE CELLS

The effect of lactate on myogenesis has been studied *in vitro* and *in vivo*. In undifferentiated C2C12 myoblasts, culture with lactate and caffeine increased the number of proliferating cells and the number of nuclei per fiber in differentiated myotubes (Oishi et al., 2015). The same study showed that the lactate effect in myoblast differentiation was accompanied by an upregulation of mTOR and P70S6K phosphorylation, suggesting that lactate may work by activating anabolic signals in SCs. Similarly, a more recent study (Ohno et al., 2019) showed that differentiated C2C12 myoblasts cultured in differentiation medium containing lactate increased the size of myotube diameters and the number of nuclei per fiber. Moreover, the study showed that in mouse, oral administration of lactate stimulated muscle hypertrophy and increased the number of Pax7-positive cells, indicating the positive effect of lactate on skeletal muscle regeneration. In contrast, another study reported that lactate promoted the early stages of C2C12 differentiation in a reactive oxygen-dependent manner, but delayed the late stages, as indicated by the number of MHC-positive nuclei (Willkomm et al., 2014).

Another lactate-dependent mechanism that indirectly directs SC fate was reported recently. By using pfkfb3-deficient mice (pfkfb3 codes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 protein, which regulates glycolysis), Zhang et al. (2020) found that lactate secreted by endothelial cells was uptaken by and regulates the polarization of macrophages, which improved muscle reperfusion in hindlimb ischemia, and therefore promoted muscle revascularization and regeneration upon SC activation (**Figure 1C**).

Another mechanism by which lactate has been shown to affect macrophage polarity (which has not been related to muscle regeneration yet) is histone lactylation in which lactate mediates

histone modifications (Zhang et al., 2019). That study correlated histone lactylation in macrophages during M1 polarization with the increased expression of genes that are involved in wound healing (i.e., genes that characterize M2 macrophages), suggesting a role in tissue homeostasis. It is unclear whether histone lactylation plays a role in skeletal muscle regeneration or if this histone modification is relevant for SC fate. Future studies should address these biological questions.

CONCLUSION

Metabolism has been shown to be closely related with SC fate. A switch from fatty acids oxidation to glycolytic metabolism accompanies the transition from quiescent to activated SCs. This metabolic switch is also related to the redox balance produced by epigenetic changes *via* the NAD+-SIRT1 pathway and ultimately directs the fate of SCs to commit to myogenic differentiation. Lactate affects myoblast differentiation. Considering this, lactate's close relationship with the redox balance and NAD+/NADH, and lactate's mediation of histone modifications, the role of lactate in the myogenic differentiation of SCs should be considered in future investigations.

AUTHOR CONTRIBUTIONS

MN conceptualized the manuscript and wrote the manuscript. MT and ZR proofread the manuscript. All authors contributed to the article and approved the submitted version.

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CORRECTION
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Corrigendum: Lactate Metabolism and Satellite Cell Fate

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A Corrigendum on

Lactate Metabolism and Satellite Cell Fate

by Nalbandian, M., Radak, Z., and Takeda, M. (2020). Front. Physiol. 11:610983. doi: 10.3389/fphys.2020.610983

In the original article, there was an error. We state that LDHA isoform converts lactate to pyruvate and that LDHB converts pyruvate into lactate. The current evidence suggests that LDHA and LDHB both can favor the lactate to pyruvate conversion in any direction.

A correction has been made to "***LACTATE AS A KEY METABOLITE IN THE CONTROL OF CELL SIGNALING," Paragraph 1***: *"Lactate is a metabolite produced from pyruvate by lactate dehydrogenase (LDH), with the LDH isoform A (LDHA) facilitating the pyruvate-to-lactate conversion in cells with high glycolytic rates, and the LDH isoform B (LDHB) facilitating the lactate-to-pyruvate conversion in highly oxidative cells. When the cytoplasmic lactate concentration is elevated, lactate can be co-transported with one H+ ion outside the cell by facilitated diffusion via monocarboxylate transporters (MCTs; Halestrap and Wilson, 2012; Kitaoka et al., 2012; Halestrap, 2013; Perez-Escuredo et al., 2016). MCT1 and MCT4 are MCT isoforms expressed in skeletal muscle (Bonen, 2001). MCT1, which has a relatively low Km (3.5–10 mM; Halestrap, 2012), is the predominant isoform in oxidative skeletal muscle fibers and considered responsible for lactate uptake (Mccullagh et al., 1997; Juel and Halestrap, 1999; Pilegaard et al., 1999; Halestrap, 2012; Chatel et al., 2017). On the other hand, MCT4, which has a much higher Km (22–28 mM; Halestrap, 2012), is the isoform predominantly expressed in glycolytic skeletal muscle fibers and considered responsible for lactate release (Dimmer et al., 2000; Fox et al., 2000; Bisetto et al., 2019). Extracellular lactate can travel through the blood stream to many cells, serving as an important energy source for several tissues and organs such as the brain (van Hall et al., 2009; Mosienko et al., 2015), liver, and skeletal muscle (Hui et al., 2017; Brooks, 2020).*

Given lactate's ability to travel between cells, tissues, and organs, recently it was proposed to be a signaling molecule (Nalbandian and Takeda, 2016; Brooks, 2020)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Elevated Lactate by High-Intensity Interval Training Regulates the Hippocampal BDNF Expression and the Mitochondrial Quality Control System

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High-intensity interval training (HIIT) is reported to be beneficial to brain-derived neurotrophic factor (BDNF) biosynthesis. A key element in this may be the existence of lactate, the most obvious metabolic product of exercise. *In vivo*, this study investigated the effects of a 6-week HIIT on the peripheral and central lactate changes, mitochondrial quality control system, mitochondrial function and BDNF expression in mouse hippocampus. *In vitro*, primary cultured mice hippocampal cells were used to investigate the role and the underlying mechanisms of lactate in promoting mitochondrial function during HIIT. *In vivo* studies, we firstly reported that HIIT can potentiate mitochondrial function [boost some of the mitochondrial oxidative phosphorylation (OXPHOS) genes expression and ATP production], stimulate BDNF expression in mouse hippocampus along with regulating the mitochondrial quality control system in terms of promoting mitochondrial fusion and biogenesis, and suppressing mitochondrial fission. In parallel to this, the peripheral and central lactate levels elevated immediately after the training. *In vitro* study, our results revealed that lactate was in charge of regulating mitochondrial quality control system for mitochondrial function and thus may contribute to BDNF expression. In conclusion, our study provided the mitochondrial mechanisms of HIIT enhancing brain function, and that lactate itself can mediate the HIIT effect on mitochondrial quality control system in the hippocampus.

Keywords: High-intensity interval training, lactate, mitochondrial quality control system, BDNF, mouse hippocampus

INTRODUCTION

High-intensity interval training (HIIT) is a type of exercise involving several sets of alternating epochs of high intensity [shorter periods of workloads near or above peak aerobic capacity (VO_{2max})] and low intensity (longer periods of rest or about 40–60% HR_{max} exercise) (Haram et al., 2009; Gibala et al., 2012; Nokia et al., 2016; Robinson et al., 2018). This pattern of exercise is more effective than conventional aerobic exercise in promoting brain health, such as cognitive function enhancement (Afzalpour et al., 2015; Saucedo Marquez et al., 2015; Nokia et al., 2016; Slusher et al., 2018; Luan et al., 2019). Both clinical and animal experiments show that HIIT can evoke higher levels of brain-derived neurotrophic factor (BDNF) production to improve the brain function in health, diabetics, and stroke patients (Afzalpour et al., 2015; Tonoli et al., 2015; Saucedo Marquez et al., 2015; Di Battista et al., 2018; Freitas et al., 2018; Boyne et al., 2019). However, it is still unclear how HIIT promotes BDNF production. One of the important underlying reason is likely to be that HIIT efficiently regulates mitochondrial quality control system for mitochondrial function enhancement, which is beneficial to improving hippocampal BDNF expression. Evidence for this is that 12-week HIIT can reverse cognitive impairment in APP/PS1 transgenic mice via promoting hippocampal mitochondrial fusion and simultaneously inhibiting mitochondrial fission (Li et al., 2019).

Mitochondrial function is mainly regulated by the mitochondrial quality control system, which includes mitochondrial fusion, fission, mitophagy, and biogenesis. Mitochondrial fusion is regulated by mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy (OPA1). MFN1 and MFN2 regulate outer mitochondrial membrane (OMM) fusion, while OPA1 orchestrates inner mitochondrial membrane fusion. These proteins act together to repair the dysfunctional mitochondria by mixing their contents with those of healthy mitochondria (Rovira-Llopis et al., 2017). Mitochondrial fission will then segregate the badly damaged mitochondria. This process is mainly regulated by cytosolic GTPase dynamin-related protein1 (DRP1) and mitochondrial fission 1 protein (FIS1) in mammals (Cai and Tammineni, 2016). DRP1 can assemble into a ring-like structure that squeezes cellular membranes to divide mitochondria into two parts. FIS1 is anchored to the OMM, and its function is to recruit DRP1 from the cytoplasm to the OMM (Flippo and Strack, 2017). In this process, the mitophagy programme is initiated to eliminate bad mitochondria, and the mitochondrial biogenesis signal is activated to renew mitochondrial production (Tatsuta and Langer, 2008; Vina et al., 2009; Bernardo et al., 2016; Zhang et al., 2016). In mitophagy, damaged mitochondria can be recognized by autophagosomes to trigger their degradation and packaging into lysosomes, which is regulated by the PTEN-induced putative kinase 1 (PINK1)-PARKIN signaling pathway (Yoon et al., 2011). Activated PINK1 recruits PARKIN for polyubiquitination, known as cytosolic E3-ubiquitin ligase. The polyubiquitinated substrates are then recognized by microtubule-associated proteins light chain 3 (LC3) adapters, such as Sequestosome-1 (P62), to interact with LC3 and recruit the tagged mitochondria to autophagosomes (Yoo and Jung, 2018).

For mitochondrial biogenesis, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is the first stimulator, and it acts as a key factor connecting several regulator cascades (Fernandez-Marcos and Auwerx, 2011; Gureev et al., 2019). It activates the nuclear respiratory factors NRF1 and NRF2. Subsequently, they activate the expression of transcription factor A (TFAM), and as a result, they increase the transcription and translation of mitochondrial DNA (mtDNA). This in turn leads to duplication of mitochondria. Briefly, these mitochondrial pathways ensure the continuous production of new mitochondria in the cell and the timely removal of old or damaged mitochondria from the cellular compartment. Therefore, the mitochondrial quality control system is essential for the maintenance of mitochondrial function, such as oxidative phosphorylation (OXPHOS) related-genes and ATP. However, to date, it is unclear how HIIT influences the hippocampal mitochondrial quality control system and mitochondrial function.

Recently, the diverse roles of lactate in mediating brain function have been brought into focus, and the positive effect of exercise on brain function has been demonstrated to be mediated by lactate (Schiffer et al., 2011; Tsukamoto et al., 2016a,b; Charalambous et al., 2018; Hashimoto et al., 2018; Malik et al., 2018; Rodriguez et al., 2018; Boyne et al., 2019). During HIIT, blood lactate levels can increase from baseline (∼2 mM in human and ∼3 mM in mouse) to 10∼15 mM in human and mouse (Morland et al., 2017; Coxon et al., 2018). The high levels of blood lactate then cross the blood-brain-barrier (BBB) via monocarboxylic acid transporter1 (MCT1) into the brain (Bergersen, 2015). Moreover, lactate can also be produced via glycolysis in astrocytes and transported by monocarboxylic acid transporter 4 (MCT4) and MCT1 into the extracellular space (Salmina et al., 2015; Takashi Matsuia et al., 2017). As a result, the level of brain lactate obviously increases (Rasmussen et al., 2011; Wiegers et al., 2017). The increased lactate can potentiate neuronal synaptic plasticity to promote learning and memory (Schurr et al., 1988; Yang et al., 2014; Margineanu et al., 2018; El Hayek et al., 2019; Scavuzzo et al., 2020), and control neuronal activity to mediate brain excitability (Bozzo et al., 2013; Tang et al., 2014; Herrera-Lopez and Galvan, 2018; de Castro Abrantes et al., 2019). In addition, lactate is likely to participate in the improvement and maintenance of brain mitochondrial function. *In vitro* studies show that ATP production for supplying neuronal energy depends on lactate (Shen et al., 2015; Vardjan et al., 2018), and lactate can also promote mitochondrial biogenesis (Hashimoto et al., 2007; Lezi et al., 2013).

Therefore, we speculate that increased hippocampal lactate immediately after HIIT may enhance the hippocampal mitochondrial function and promote BDNF expression via regulating mitochondrial quality control system. To test this hypothesis, we use both vivo and vitro study and identify a hitherto-unknown role of lactate. We highlight that HIIT, as a non-invasive stimulating manner, represents a promising strategy to boost brain fitness. Furthermore, ingesting 10∼20 mM lactate to replace strenuous exercise (above the lactate threshold),

at a safe and effective physiological level, maybe a great strategy for people in urgent need of ameliorating brain function incapable of exercise.

MATERIALS AND METHODS

Animals

Male mice were 7 weeks of age at the start of the experiments and procured from Nanjing Model Animal Research Center (Certificate SCXK 2018-0008). They were housed in cages and provided with food and water *ad libitum*. The room temperature was maintained at (22–24)°C under a 12 h light-dark cycle. After 1 week acclimatization period, mice were divided randomly into two groups (n = 22 each group): sedentary control group (Ctl group) and HIIT group. Mice were anesthetized with diethyl to sacrifice within 1 h (for the hippocampal lactate detection) or 24 h (for the other indicators detection) after 6-week exercise paradigm. The hippocampus was dissected on crushed ice immediately and then stored at −80°C for later analysis. Animal care and use were in accordance with the guidelines set by the Institutional Animal Ethics Committee (IAEC) and as approved by the Ethical Committee for Science Research at Shanghai (102772019DW010).

HIIT Paradigm

Mice in the HIIT group were familiarized with five bouts of treadmill running (4 min × (20–22) m/min) and active rest (2 min × 10 m/min) for 5 days. After familiarization, the mice completed 6-week HIIT. They were exposed to the HIIT exercise protocol for five consecutive days each week. This exercise regime has been previously described (Morland et al., 2017). Briefly, each session consisted of warm-up (10 min × 10 m/min), followed by 10 bouts of 4 min high-intensity running (4 min × (80–90)% $Speed_{max}$), and separated by active rest (2 min × (40–50)% $Speed_{max}$). Running took place on a treadmill at a 0 degree (**Figure 1A**). The maximal speed test for mice was, respectively, performed at the end of adaptive training, the second and the fourth week of training, and adjust the speed of interval training (**Figure 1B**). In this regard, the beginning speed of mice was 10 m/min for lasting 10 min to warm up. Then the speed was increased by 2 m/min every 2 min until exhaustion. Throughout the experiment, the Ctl group did not receive any exercise training.

Primary Hippocampal Cell Culture

Primary hippocampal cells were obtained from 1-day-old neonatal C56BL/6J mice as previously described (Cai et al., 2020), which were purchased from Shanghai JSJ Lab Animal Center (Certificate SCXK 2013-0016, Shanghai, China). Briefly, the dissociated hippocampal tissues were collected in ice-cold D-Hanks' balanced salt solution (HBSS; No. H6648, Sigma-Aldrich, United States) and digested into a single cell suspension in 0.25% trypsin/EDTA (No. 25200072, Gibco, United States) for 10–15 min. Termination of the digestion is supplemented with Dulbecco's Modified Eagle Medium (DMEM; No. 11960044, Gibco, United States), which contained 10% Fetal Bovine Serum (No. 10099141, Gibco, United States). Primary hippocampal cells (1.0×10^6 cells/ml) were maintained with serum-free Neurobasal Medium (No. 10888022, Gibco, United States) containing 2% B27 (No. 17504044, Gibco, United States) and 1% penicillin-streptomycin (No. C0222, Beyotime, Shanghai, China) in 6-well plates, which were previously coated with 0.01% poly-L-lysine (No. P4832, Sigma-Aldrich, United States). Hippocampal cells were cultured in a humidified incubator at 37°C with 5% CO_2 with a half- medium changed every 2 days. After 7 days, the cells were harvested for the follow-up experiments.

Cell Treatment

On 5th day, cells were treated with different concentrations (5, 10, 15, and 20 mM) of sodium L- lactate (No. L7022, Sigma-Aldrich, United States) for 3, 6, 12, and 24 h at the incubator. Lactate was prepared as 20 mM stock in configured Neurobasal medium before using.

Lactate Measurements

Blood lactate was detected by Lactate-Scout (EKF Co., Germany) immediately at the end of 2nd, 4th, and 6th week after completing the 10 bouts of training. Ten of all mice in each group were collected from tail tip blood. Hippocampal lactate level was measured using the method described by El Hayek et al. (2019). At the end of the 6-week training and after the anesthetization, 20 mice (n = 10 each group) were sacrificed immediately. Hippocampal lactate levels were measured using the L-lactate assay kit (No. ab65331, Abcam) according to the manufacturer's protocol. We used TCA Kit (No. ab204708, Abcam) to remove enzyme from tissue sample in case of endogenous LDH degrading lactate.

Western Blotting

At the end of the 6-week training, 24 h after the last training and after the anesthetization, all mice were sacrificed. The left hippocampus of each mouse brain was carefully dissected out following the standard procedure. The hippocampus of each mice (six animals per group randomly) was washed with pre-cooling PBS. The sample was homogenated at 14,000 g for 5 min in RIPA Lysis Buffer (No. P0013B, Beyotime, Shanghai, China), which contained phosphatase and protease inhibitors, with 1 mM PMSF (No. ST506, Beyotime, Shanghai, China), and the supernatant was collected. Material from vitro experiments was processed similarly. Proteins (30 μg) in SDS were loaded on a 10–12.5% polyacrylamide gradient gel. The gel was then transferred to 0.22 μm PVDF membrane (Epizyme Biotech, Shanghai, China). The membranes were blocked with Protein Free Rapid Blocking Buffer (No. PS108P Epizyme Biotech, Shanghai, China) for 10 min and then incubated for 10 h at 4°C with primary antibodies. After washing, the membranes were incubated for 1 h with secondary antibodies. Quantification of the band density was performed using the ImageJ. Protein levels were normalized to the band intensity of TUBULIN. Details of the antibodies used are provided below (**Table 1**).

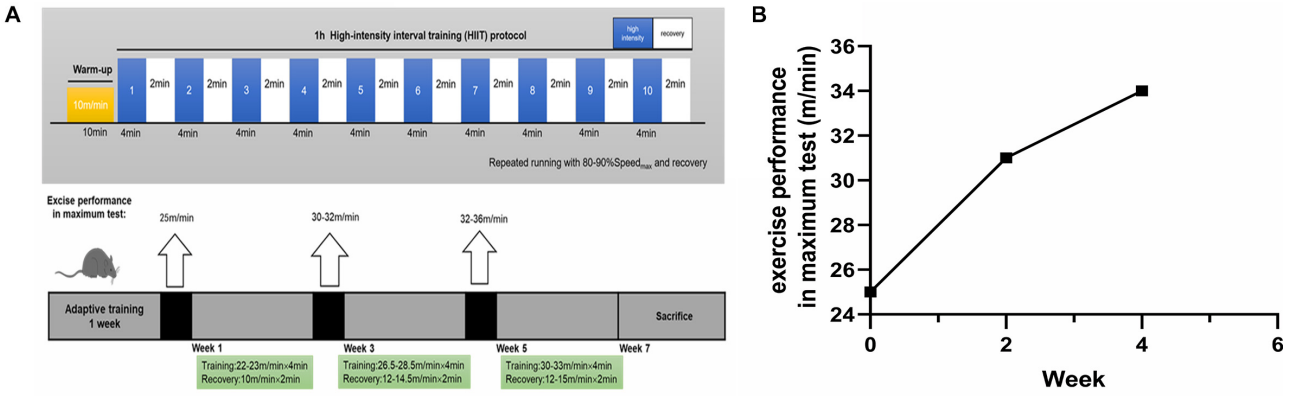

FIGURE 1 | The exercise paradigm of 6-week HIIT and exercise performance in the maximum test. **(A)** The exercise paradigm of 6-week HIIT. In the first and second week, the HIIT group completed warm-up (10 min × 10 m/min) and then 10 bouts of high-intensity running [4 min × (22–23) m/min], and separated by active rest (2 min × 10 m/min). In the third and fourth week, the HIIT group completed warm-up and then 10 bouts of high-intensity running [4 min × (26.5–28.5) m/min], and separated by active rest [2 min × (12–14.5) m/min]. In the fifth and sixth week, the HIIT group completed warm-up and then 10 bouts of high-intensity running [4 min × (30–33) m/min], and separated by active rest [2 min × (12–15) m/min]. **(B)** The exercise performance in the maximum test. The exercise performance of the HIIT group was, respectively, performed at the end of adaptive training, the second and the fourth week of training.

TABLE 1 | Antibodies used for WB analysis of mice proteins.

Antibody name	Molecular weight (kDa)	Classification	Antibody source	Catalog number	Dilution ratio
MFN1	84	mitochondrial fusion	Proteintech	13798-1-AP	1:2000
MFN2	80		CST	9482	1:1000
OPA1	80–100		CST	80471	1:1000
DRP1	82	mitochondrial fission	CST	8570S	1:1000
FIS1	17		Proteintech	10956-1-AP	1:1000
PINK1	66	mitochondrial autophagy	Abcam	ab23707	1:1000
PARKIN	50		CST	4211	1:1000
P62	62		MBI	PM045	1:1000
LC3II/I	16, 14		CST	12741	1:1000
PGC-1α	91	mitochondrial biogenesis	Novus	NBP1-04676	1:1000
NRF1	68		CST	46743	1:1000
NRF2	97		CST	12721	1:1000
TFAM	28		Abcam	ab131607	1:2000
MCT1	54	monocarboxylic acid transporter	Abcam	ab90582	1:1000
MCT4	49		Proteintech	22787-1-AP	1:1000
B-TUBULIN	50		Proteintech	10068-1-AP	1:2000

RNA Extraction and Real-Time PCR (RT-PCR)

Total RNA was prepared from right hippocampal tissues or primary cells using TRIZOL (Ambion, United States). The RNA quality was assessed on a NanoDrop 2000 (Thermo, United States), where the 260/280 ratio was obtained. Samples with a ratio of 1.8–2.0 were processed for gene analysis. Reverse transcription was performed using PrimeScript^{TM}RT Master Mix (No. RR036A, Takara) according to the manufacturer's protocol. Real-time PCR was performed using SYBR® Premix Ex Taq II (No. RR820A, Takara) and StepOnePlus Real-Time PCR System (Applied Biosystems, CA, United States). The mRNA levels were normalized to the internal loading control of GAPDH and determined with the comparative ΔΔCT method. OXPHOS related genes include NDUFS8, SDHb, Uqcrc1, COX5b, and Atp5a1. Details of the Primers used are provided below (**Table 2**).

gDNA Extraction and RT-PCR

gDNA was prepared from left hippocampal tissues or primary cells using Takara MiniBEST Universal Genomic DNA Extraction Kit (No. 9765, Takara) according to the manufacturer's instructions. The DNA quality was assessed on a NanoDrop 2000 (Thermo), where the 260/280 ratio was obtained. Samples with a ratio of 1.8–2.0 were processed for gene analysis. RT-PCR were performed using SYBR® Premix Ex Taq II (No. RR820A, Takara). The mitochondrial DNA copy number was determined by RT-PCR using primers specific for cytochrome B (mt-Cytb)- a mitochondrial genome encoded gene and was normalized to levels of a nuclear-encoded gene cytochrome C (Cycs). Relative

TABLE 2 | Primer sequences used for RT-PCR analysis of mice cDNA.

Gene name	Primer	Sequence (5′–3′)
NDUFS8	Forward	AGTGGCGGCAACGTACAAG
	Reverse	TCGAAAGAGGTAACTTAGGGTCA
SDHb	Forward	AATTTGCCATTTACCGATGGGA
	Reverse	AGCATCCAACACCATAGGTCC
Uqcrc1	Forward	ACGCAAGTGCTACTTCGCA
	Reverse	CAGCGTCAATCCACACTCCC
COX5b	Forward	TCTAGTCCCGTCCATCAGCAAC
	Reverse	GCAGCCAAAACCAGATGACAGT
Atp5a1	Forward	TCTCCATGCCTCTAACACTCG
	Reverse	CCAGGTCAACAGACGTGTCAG
MCT1	Forward	TGTTAGTCGGAGCCTTCATTTC
	Reverse	CACTGGTCGTTGCACTGAATA
MCT4	Forward	TCACGGGTTTCTCCTACGC
	Reverse	GCCAAAGCGGTTCACACAC
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA

TABLE 3 | Primer sequences used for mtDNA analysis of mice.

Gene name	Primer	Sequence (5′–3′)
mt-Cytb	Forward	TGCATACGCCATTCTACG
	Reverse	ATGGGTGTTCTACTGGTTG
Cycs	Forward	CAGTGCAGAATTACCAGGTGTG
	Reverse	GGTCTGCCCTTTCTCCCTTCT

mitochondrial DNA levels between groups were quantified by comparative ΔΔCT method as previously described (Guo et al., 2009; Fanibunda et al., 2019). Details of the Primers used are provided below (**Table 3**).

ATP Detection

The ATP levels in the right hippocampus and primary cells were measured using the method described by Sheng et al. (2009) according to the manufacturer's instruction (No. S0026, Beyotime, Shanghai, China). Briefly, the hippocampal tissue or harvested cells were lysed with ATP lysis buffer and then centrifugated at 12,000 *g* for 5 min at 4°C. A total of 20 μl supernatant was mixed with 100 μl luciferase reagent in a lighttight microplate, which was measured by Luminance. ATP levels were normalized to the protein content of each sample, estimated using a BCA protein assay kit (No. P0010, Beyotime, Shanghai, China) and expressed as fold change of treated over control.

Statistics

All data are presented as means ± SEM. *In vivo* study, comparisons of two groups were performed using an unpaired Student's *t* test. *In vitro* study, comparisons of two groups were performed using non-parametric Mann-Whitney. Comparisons of multiple groups were performed using non-parametric Kruskal-Wallis *H* test. A *p* value of 0.05 or less was considered statistically significant. All calculations and the graph construction were performed using SPSS 20.0 and GraphPad 8.0 software (La Jolla, CA, United States).

RESULTS

HIIT Increased Lactate Levels and Promoted Hippocampal MCT1/4 and BDNF Expression

The blood lactate was detected at the end of 2nd, 4th, and 6th week of training to estimate the exercise intensity. We found that the blood lactate levels in HIIT group can reach to 6∼10 mM immediately after training at the end week of 2nd (6.6 ± 0.44 mM in the HIIT group vs. 2.67 ± 0.14 mM in the Ctl group, $t(10.81) = -8.43$, $p < 0.01$), 4th (7.07 ± 0.54 mM in the HIIT group vs. 4.13 ± 0.25 mM in the Ctl group, $t(18) = -4.89$, $p < 0.01$) and 6th (9.15 ± 0.68 mM in the HIIT group vs. 3.32 ± 0.20 mM in the Ctl group, $t(18) = -8.21$, $p < 0.01$), which are significantly increased compared with the Ctl group, respectively (**Figure 2A**). The hippocampal lactate levels in HIIT group were also significantly higher than those in the Ctl group (1.90 ± 0.52 mM in the HIIT group vs. 1.39 ± 0.25 mM in the Ctl group, $t(12.91) = -2.81$, $p = 0.02$, **Figure 2B**), the results suggested that this high-intensity exercise regime can effectively increase the lactate levels. Moreover, HIIT promoted mRNA and protein expression of MCT1 and MCT4 in mouse hippocampus ($t(10) = -5.14$, $p < 0.01$ for MCT1 protein; $t(10) = -3.50$, $p = 0.01$ for MCT1 mRNA; $t(10) = -5.86$, $p < 0.01$ for MCT4 protein; $t(10) = -3.02$, $p = 0.01$ for MCT4 mRNA, **Figures 2C,E–H**), suggesting that the blood lactate and astrocytic glycogen-derived lactate were transported into intercellular space for hippocampal lactate increase. Meanwhile, the mice hippocampal BDNF expression was higher in the HIIT group than the Ctl group ($t(10) = -6.102$, $p < 0.01$ **Figures 2C,D**).

The Effects of HIIT on Hippocampal Mitochondrial Function

The levels of hippocampal mitochondrial OXPHOS related genes (NDUFS8, SDHb, Uqcrc1, COX5b, Atp5a1) expression and ATP were used to reflect mitochondrial function. The results showed that HIIT significantly promoted COX5b gene expression ($t(5.27) = -3.08$, $p = 0.03$, **Figure 3B**), but there was no significant difference in the gene expression of NDUFS8, SDHb, Uqcrc1, Atp5a1. Meanwhile, we found that HIIT notably elevated hippocampal ATP levels ($t(10) = -2.67$, $p = 0.02$, **Figure 3A**). The above results demonstrated that HIIT had the potential to enhance hippocampal mitochondrial function.

The Effects of HIIT on Hippocampal Mitochondrial Fusion and Fission

Mitochondrial function is mainly influenced by the mitochondrial quality control system, fusion and fission are of important components. The results showed that HIIT promoted the fusion protein expression of OPA1, MFN1, and MFN2 ($t(5.64) = -3.75$, $p = 0.01$; $t(7.13) = -2.74$, $p = 0.03$; $t(10) = -5.38$, $p < 0.01$, **Figures 4E–G**), but significantly

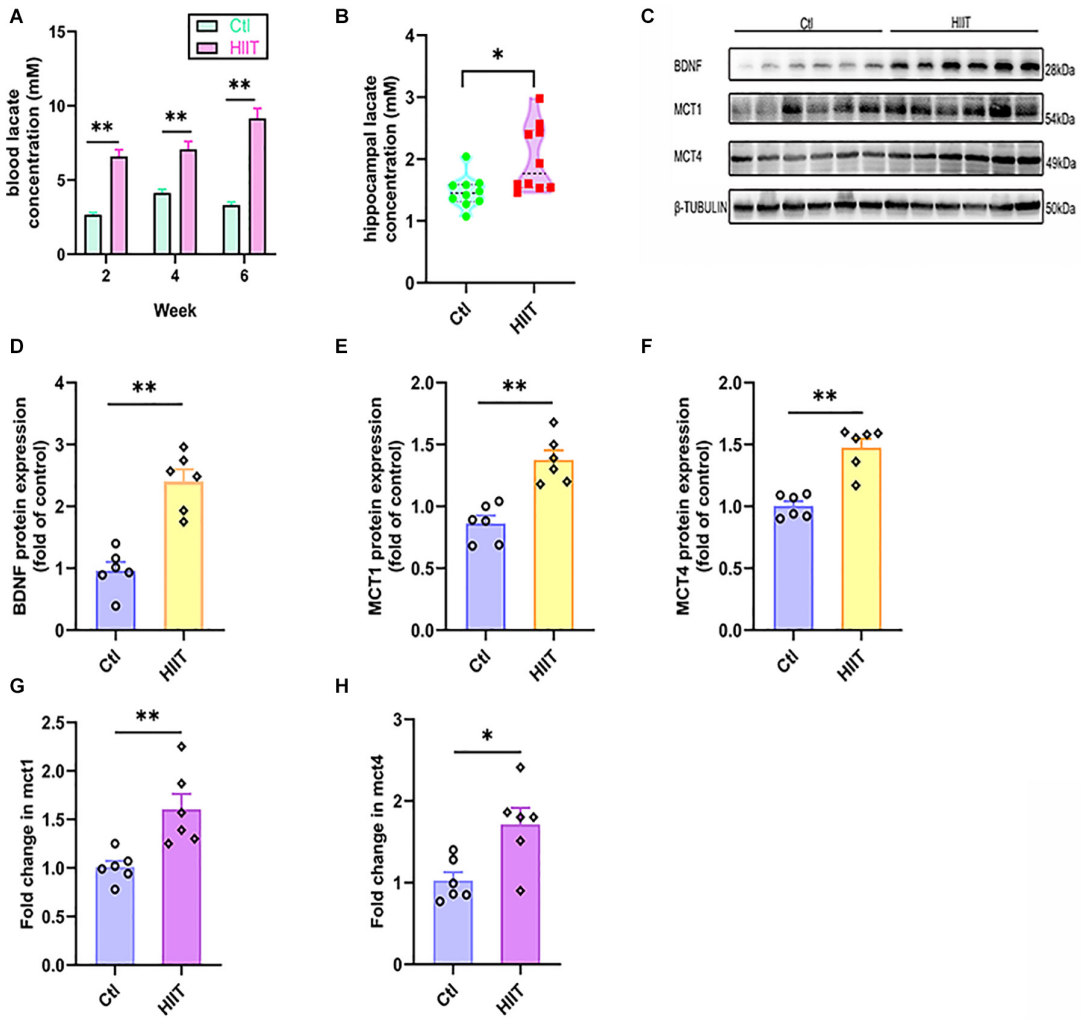

FIGURE 2 | HIIT increased lactate levels and promoted hippocampal MCT1/4 and BDNF expression. The blood lactate levels in two groups were detected at the end of 2nd, 4th, and 6th week of training to estimate the exercise intensity of High-intensity interval training (HIIT). The hippocampal lactate level was determined by colorimetry. The levels of Monocarboxylic acid transporter 1 (MCT1) and Monocarboxylic acid transporter 4 (MCT4) were also detected by RT-PCR and WB, respectively, to indirectly evaluate the lactate level in mouse hippocampus. The protein expression of Brain-derived neurotrophic factor (BDNF) was detected by WB. **(A)** The blood lactate levels. **(B)** hippocampal lactate level. **(C)** Representative WB image. **(D)** BDNF protein expression. **(E)** MCT1 protein expression. **(F)** MCT4 protein expression. **(G)** mct1 mRNA level. **(H)** mct4 mRNA level. TUBULIN and gapdh as the loading control. *N* = 10 mice per group for **(A,B)**. *N* = 6 mice per group for **(D-H)**. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ as compared with the Ctl group by unpaired Student's *t* test. Values are expressed as mean ± standard error of the mean.

inhibited the fission protein expression of DRP1 and FIS1 ($t(6.64) = 2.87$, $p = 0.03$; $t(10) = -3.56$, $p = 0.01$, **Figures 4A–D**). These results demonstrated that the significant increase in the above oxidative phosphorylation levels in hippocampus may be due to the enhanced mitochondrial fusion during the HIIT.

The Effects of HIIT on Hippocampal Mitophagy

As an acute tissue stress response occurred, mitochondrial fusion and fission are often accompanied with mitophagy. The PINK1 mediated mitophagy signals can be activated to remove the damaged mitochondria. Our results showed that there was no significant difference between the two groups in protein expression of PINK1, PARKIN, and LC3II/I (**Figures 5A–C,E**), although the protein expression of P62 was decreased in HIIT group compared to Ctl group ($t(10) = 3.97$, $p < 0.01$, **Figures 5A,D**). The results suggested that HIIT had little effect on mice hippocampal mitophagy, namely, HIIT didn't damage hippocampal mitochondria.

The Effects of HIIT on Hippocampal Mitochondrial Biogenesis Signal and Mitochondria DNA Copy Number

Mitochondrial biogenesis is critical for generating new mitochondria and maintaining mitochondrial homeostasis, which is orchestrated by PGC-1α-regulated signaling pathway.

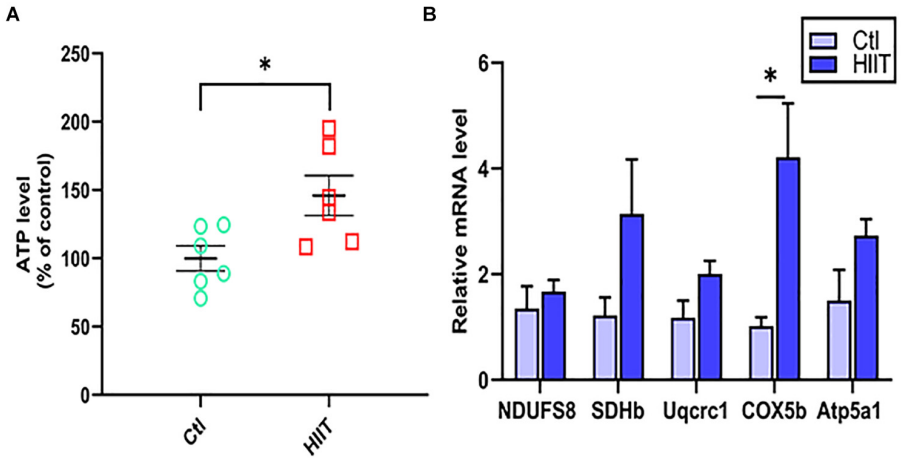

FIGURE 3 | The effects of HIIT on hippocampal mitochondrial function. The level of ATP was detected by Luciferase and the oxidative phosphorylation (OXPHOS) related genes (NDUFS8, SDHb, Uqcrc1, COX5b, and Atp5a1) was detected by RT-PCR in mouse hippocampus. **(A)** The ATP level. **(B)** OXPHOS mRNA levels. The gapdh as the loading control. $N = 6$ mice per group. $^{*}P \leq 0.05$ as compared with the Ctl group by unpaired Student's t test. Values are expressed as mean ± standard error of the mean.

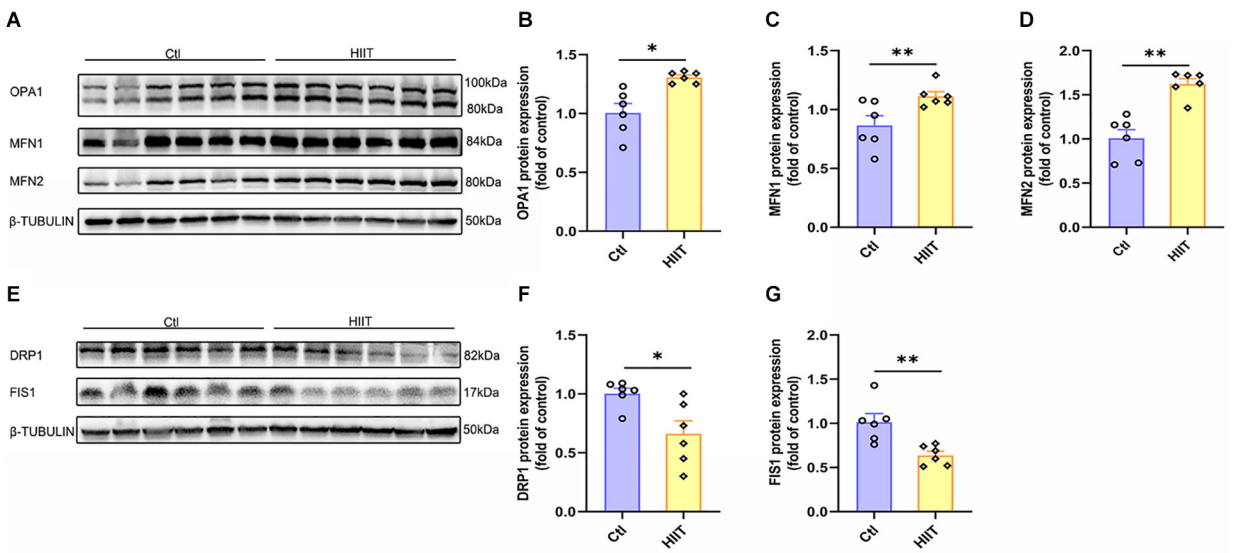

FIGURE 4 | The effects of HIIT on hippocampal mitochondrial fusion and fission. The Optic atrophy (OPA1), Mitofusin 1 (MFN1), and Mitofusin 2 (MFN2) were determined by WB to represent the mitochondrial fusion state in mouse hippocampus. The Dynamin-related protein1 (DRP1) and mitochondrial fission 1 protein (FIS1) were also determined by WB to represent the mitochondrial fission state in mouse hippocampus. **(A,E)** Representative WB image. **(B)** OPA1 protein expression. **(C)** MFN1 protein expression. **(D)** MFN2 protein expression. **(F)** DRP1 protein expression. **(G)** FIS1 protein expression. TUBULIN as the loading control. $N = 6$ mice per group. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ as compared with the Ctl group by unpaired Student's t test. Values are expressed as mean ± standard error of the mean.

PGC-1α activates TFAM by interacting with NRF1 and NRF2, causing mitochondrial DNA replication and transcription. We found HIIT promoted hippocampal PGC-1α and NRF2 protein expression ($t(5.78) = -3.49$, $p = 0.01$; $t(6.17) = -5.13$, $p < 0.01$, **Figures 6A,B,D**), accompanied by the mitochondrial copy number increased significantly ($t(5.66) = -4.24$, $p = 0.01$, **Figure 6F**). However, the protein expression of NRF1 and TFAM had no significant difference (**Figures 6A,C,E**). These results demonstrated that HIIT could facilitate hippocampal mitochondrial biogenesis signal and increase mitochondrial copy number to accommodate high energy requirements.

Lactate Increased the OXPHOS Related Genes Expression and ATP Levels in Primary Hippocampal Cells

To assess whether lactate, a metabolite produced by HIIT, directly regulates mice hippocampal mitochondrial function, we

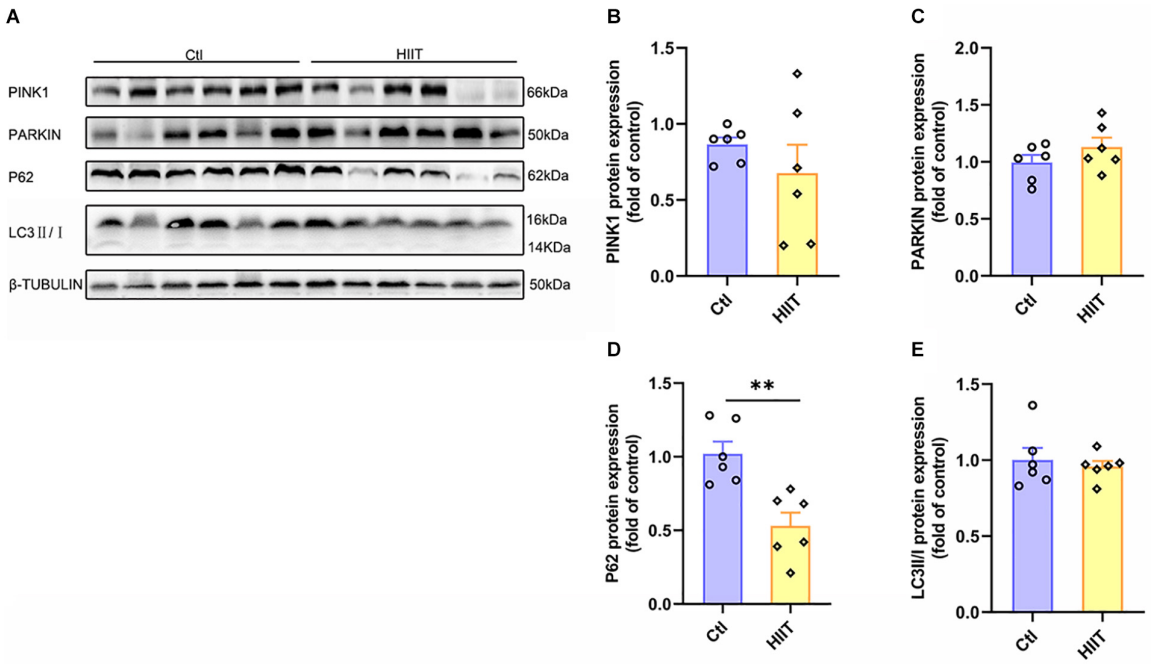

FIGURE 5 | The effects of HIIT on hippocampal mitophagy. The PTEN-induced putative kinase 1 (PINK1), PARKIN, Sequestosome-1 (P62), and microtubule-associated protein light chain3 (LC3) were determined by WB to represent the mitophagy state in mouse hippocampus. **(A)** Representative WB image. **(B)** PINK1 protein expression. **(C)** PARKIN protein expression. **(D)** P62 protein expression. **(E)** LC3 protein expression. TUBULIN as the loading control. $N = 6$ mice per group. $^{**}P \leq 0.01$ as compared with the Ctl group by unpaired Student's t test. Values are expressed as mean ± standard error of the mean.

adopted primary mice hippocampal cell culture with lactate treatment *in vitro* and detected OXPHOS subunits expression and ATP levels. We firstly detected the ATP levels with different concentrations of lactate (5, 10, 15, and 20 mM) for 24 h. The ATP levels were evoked by lactate stimulus from 15 to 20 mM ($H = 12.43$, $p = 0.01$, **Figure 7A**). The 15 mM ($p = 0.01$) and 20 mM ($p = 0.02$) lactate treatment are significantly to the 0 mM lactate treatment. Then we detected the cellular ATP level with 15 mM lactate for different time points (0, 3, 6, 12, and 24 h) ($H = 9.83$, $p = 0.04$ **Figure 7B**). The ATP levels were increased as early as 3 h and reached to the peak at 6 h after lactate treatment ($p = 0.05$, $p = 0.01$, **Figure 7B**). Based on this, we adopted the 15 mM lactate treatment for 3 h in the subsequent experiment. Then we found the gene levels of Uqcrc1 and Atp5a1 (representing complex III and ATP synthase, respectively) significantly increased after lactate treatment ($Z = -1.96$, $p = 0.05$; $Z = -1.99$, $p = 0.046$, **Figure 7C**), and there was an increased tendency in the levels of the gene NDUFS8, SDHb, and COX5b. The results confirmed that a high concentration of lactate could partly enhance the mitochondrial ability of OXPHOS related genes expression and augment ATP levels in the primary hippocampal cells.

Lactate Increased BDNF Expression in Primary Hippocampal Cells

In order to understand the role of lactate in HIIT promoting mice hippocampal BDNF expression, we detected the protein expression of BDNF in the condition of lactate treatment in mice hippocampal cells. Our result showed that the BDNF protein expression increased in the mice hippocampal cells following the lactate treatment ($Z = -1.96$, $p = 0.05$, **Figures 8A,B**).

Lactate Directly Regulated Mitochondrial Fusion and Fission in Primary Hippocampal Cells

To further determine the mechanism of lactate enhancing the mitochondrial function and the role of lactate in HIIT-mediated mitochondrial quality control system in mouse hippocampus, the mitochondrial fusion and fission biomarker proteins expression were examined in primary cultured hippocampal cells following lactate treatment. Results showed that the expression of mitochondrial fusion protein MFN1 and MFN2 was significantly upregulated ($Z = -1.96$, $p = 0.05$; $Z = -1.96$, $p = 0.05$, **Figures 9A,C,D**), but the expression of OPA1 had no difference between the two groups (**Figures 9A,B**). Meanwhile, the expression of mitochondrial fission protein DRP1 and FIS1 was inhibited after lactate treatment ($Z = -1.96$, $p = 0.05$; $Z = -1.96$, $p = 0.05$, **Figures 9E–G**). These results verified that a high concentration of lactate could promote mitochondrial fusion and suppress mitochondrial fission.

Lactate Weakly Influenced Mitophagy in Primary Hippocampal Cells

After lactate treatment, the results showed that there was no difference between the two groups in the mitophagy

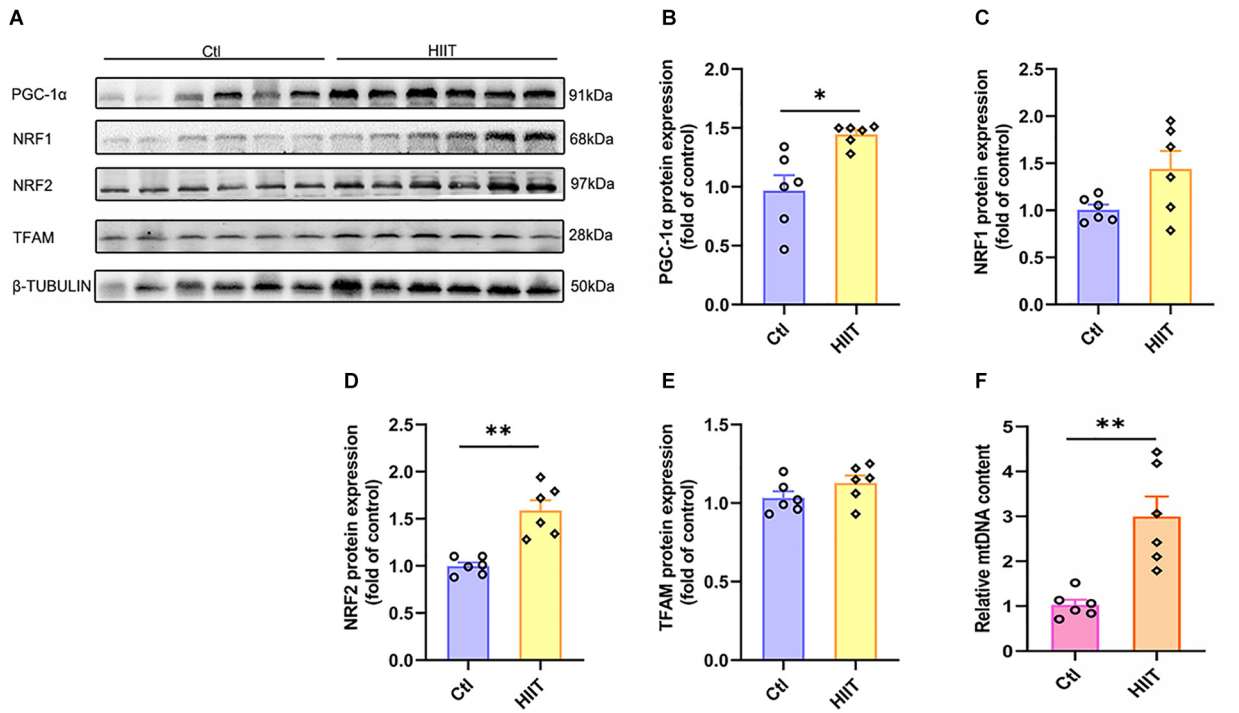

FIGURE 6 | The effects of HIIT on hippocampal mitochondrial biogenesis signal and mitochondria DNA copy number. The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factors 1 (NRF1), nuclear respiratory factors 1 (NRF2), and transcription factor A (TFAM) were determined by WB to evaluate the mitochondrial biogenesis signals in mouse hippocampus. The mt-Cytb level was determined by RT-PCR to evaluate the mitochondria DNA copy number. **(A)** Representative WB image. **(B)** PGC-1α protein expression. **(C)** NRF1 protein expression. **(D)** NRF2 protein expression. **(E)** TFAM protein expression. **(F)** mt-Cytb level. TUBULIN and Cycs as the loading control. $N = 6$ mice per group. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ as compared with the Ctl group by unpaired Students *t* test. Values are expressed as mean ± standard error of the mean.

related proteins expression of PINK1, PARKIN, and P62 (**Figures 10A–D**), except for the significantly downregulated protein expression of LC3II/I in the HIIT group ($Z = -1.96$, $p = 0.05$, **Figures 10A,E**). The results demonstrated that a high concentration of lactate had little effect on mitophagy, which were consistent with *in vivo* results.

Lactate Enhanced Mitochondrial Biogenesis Signal and Increased Mitochondria DNA Copy Number in Primary Hippocampal Cells

PGC-1α, NRF1, NRF2, TFAM, and mitochondria DNA copy number were detected for evaluating the regulatory effect of lactate on mitochondrial biogenesis in primary cultured hippocampal cells. We found that both the protein expression of PGC-1α, NRF2, and TFAM ($Z = -1.96$, $p = 0.05$; $Z = -1.96$, $p = 0.05$; $Z = -1.96$, $p = 0.05$, **Figures 11A,B,D,E**) and the mitochondria DNA copy number ($Z = -1.96$, $p = 0.05$, **Figure 11F**) were increased after lactate treatment, except for the NRF1 protein expression (**Figure 11C**). The results verified that a high concentration of lactate could facilitate mitochondrial biogenesis and increase mitochondria DNA copy number, which was also consistent with *in vivo* results.

DISCUSSION

High-intensity interval training is a time-efficient alternative to moderate- or low-intensity continuous exercise for improving a range of physiological indexes associated with human health. HIIT has been widely used in public fitness, weight loss, and metabolic diseases treatment and rehabilitation (Kemi et al., 2005; Weston et al., 2014; Sperlich et al., 2017; Fealy et al., 2018). Beyond these, recent studies prove the protective effect of HIIT on brain function, especially in promoting BDNF expression (Saucedo Marquez et al., 2015; Tonoli et al., 2015; Boyne et al., 2019). Our results also confirmed that HIIT could promote hippocampal BDNF expression in healthy mice, which suggests HIIT has a protective effect on the brain function. Nevertheless, the mechanism of how HIIT influencing BDNF expression remains to be investigated.

High-intensity interval training is a typical anaerobic exercise, and glycolysis is the main energy supply in this process. Therefore, lactate level increases are the most obvious metabolic changes stimulated by HIIT and can be absorbed into neurons with the help of monocarboxylic acid transporters. MCT1 is primarily located in the endotheliocyte of the blood-brain barrier where it transports blood lactate into the brain (Gerhart et al., 1997; Pierre et al., 2000), and also so well as MCT4, located at the astrocytic membranes to transport intracellular lactate,

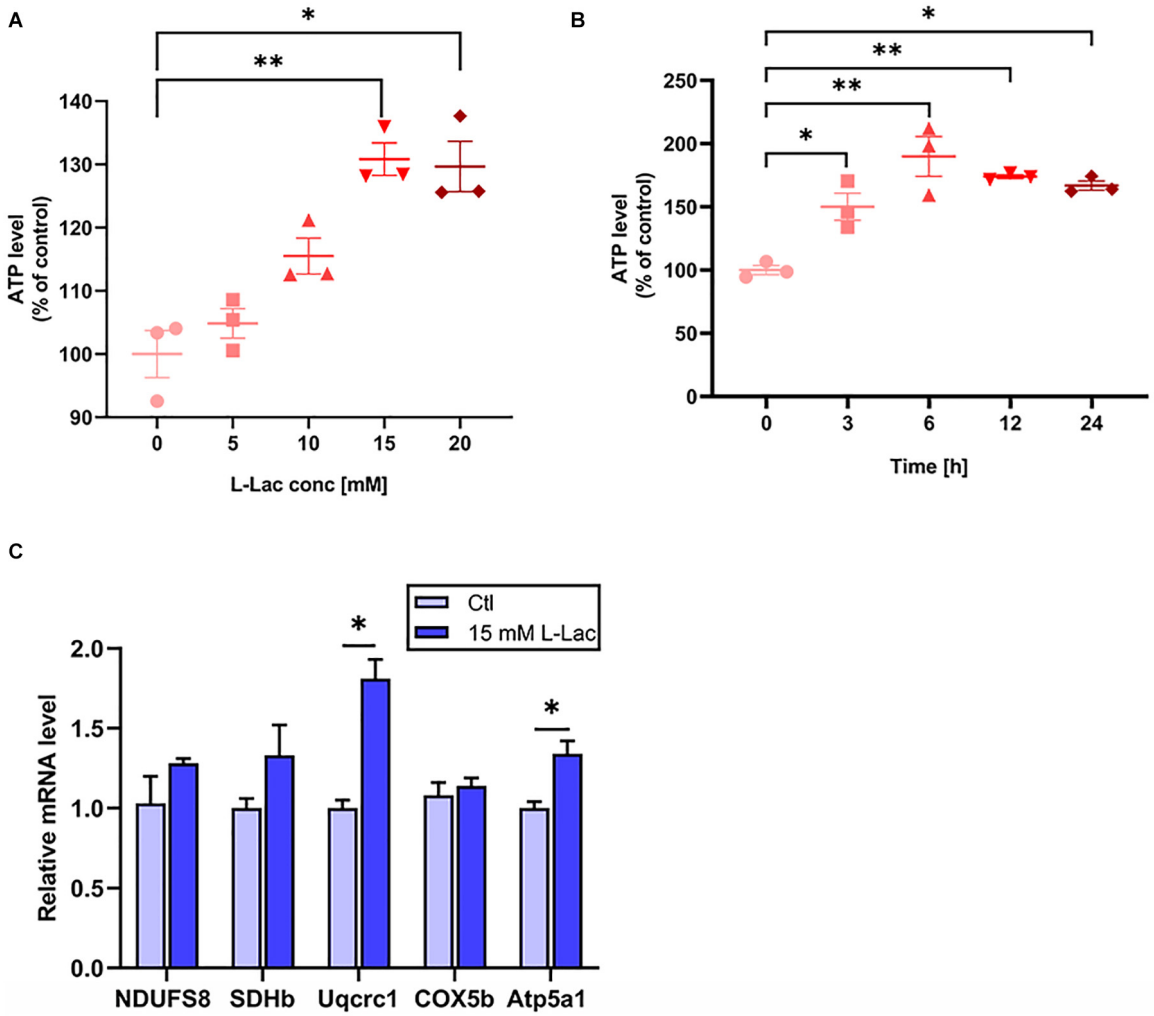

FIGURE 7 | Lactate increased the OXPHOS related genes expression and ATP levels in primary hippocampal cells. The mice primary cultured hippocampal cells were treated with different concentration of L-lactate (5, 10, 15, and 20 mM) for 24 h and different time points (3, 6, 12, and 24 h) following 15 mM lactate treatment for determination of the effect of lactate on the levels of ATP. The levels of ATP were detected by Luciferase. The oxidative phosphorylation (OXPHOS) related genes (NDUFS8, SDHb, Uqcrc1, COX5b, and Atp5a1) was detected in the condition of 15 mM L-lactate treatment for 3 h by RT-PCR in primary hippocampal cells. **(A,B)** The ATP levels. **(C)** OXPHOS mRNA levels. The gapdh as the loading control. $N = 3$ in three independent experiments. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ as compared with the Ctl group by non-parametric tests. Values are expressed as mean ± standard error of the mean.

produced by astrocytic glycolysis (Rafiki et al., 2003; Pierre and Pellerin, 2005; Bergersen, 2015). In an *in vivo* study, we found that HIIT promoted the mRNA and protein expression of MCT1 and MCT4, which may be the key reason for the increased hippocampal lactate levels. We also found that the level of blood lactate significantly increased to 6–10 mM in the HIIT group immediately after the training, while the blood lactate level was 2–4 mM in the Ctl group. Accordingly, hippocampal lactate increased (1.9 mM in the HIIT group vs. 1.4 mM in the Ctl group) immediately at the end of the exercise. Recently, the diverse roles of lactate in mediating brain function have been well characterized (Berthet et al., 2009; Lauritzen et al., 2014; Tang et al., 2014; Mosienko et al., 2015; Dienel, 2017; Magistretti and Allaman, 2018), such as mediating synaptic plasticity (Schurr et al., 1988; Yang et al., 2014; Margineanu et al., 2018; El Hayek et al., 2019; Scavuzzo et al., 2020), cerebral microvasculogenesis (Morland et al., 2017; Boitsova et al., 2018), and neuronal activity (Bozzo et al., 2013; Tang et al., 2014; Herrera-Lopez and Galvan, 2018; de Castro Abrantes et al., 2019). Therefore, we speculated that the lactate might be an intermediary between HIIT and increased hippocampal BDNF expression. Although a low sample size limits the evaluation of the positive effect of lactate on BDNF, our results show that 15 mM lactate can induce the BDNF protein expression, which is in line with the previous studies (Margineanu et al., 2018; El Hayek et al., 2019). Therefore, we think that lactate may be one of the most important factors contributing to the positive effect of HIIT on the BDNF expression in the mouse hippocampus.

Of note, BDNF biosynthesis requires a great deal of ATP (Gomez-Pinilla, 2008; Vignoli and Canossa, 2017;

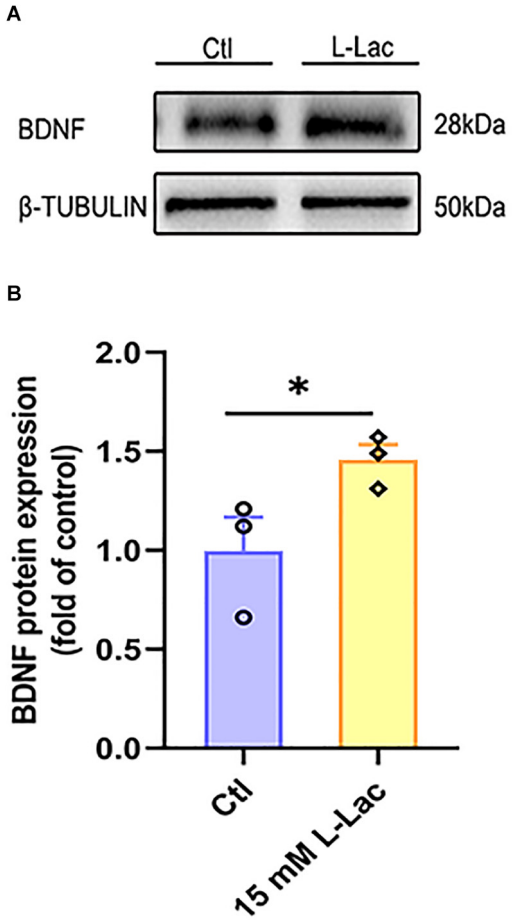

FIGURE 8 | Lactate increased BDNF expression in primary hippocampal cells. The mice primary cultured hippocampal cells were treated with 15 mM L-lactate for 3 h to observe the effect of lactate on BDNF expression. The protein expression of Brain-derived neurotrophic factor (BDNF) was detected by WB. **(A)** Representative WB image. **(B)** BDNF protein expression. TUBULIN as the loading control. $N = 3$ in three independent experiments. $^*P \leq 0.05$ compared with the Ctl group by non-parametric tests. Values are expressed as mean ± standard error of the mean.

Hu et al., 2020), and 95% of ATP is derived from mitochondrial oxidative phosphorylation (OXPHOS) (Nakamura and Lipton, 2017; Ould Amer and Hebert-Chatelain, 2018). Four large protein complexes (I, II, III, and IV) and ATP synthase in the inner mitochondrial membrane are the pivotal links that affect the efficiency of ATP production by OXPHOS. The three proton-pumping complexes of the electron transfer chain are complexes I, III, and IV. Complex II does not pump protons but contributes reduced ubiquinone. The flow of protons back into the matrix via a proton channel in the ATP synthase leads to conformational changes in the nucleotide-binding pockets and the formation of ATP (Kühlbrandt, 2015). Previous studies found that exercise, even moderate-intensity exercise (Bayod et al., 2011; Masaki Takimoto, 2014) or high-intensity exercise (Lezi et al., 2014), can increase brain mitochondrial OXPHOS. Our research results showed that HIIT promoted hippocampal COX5b gene (complex IV) expression and that a high concentration of lactate enhanced cultured hippocampal cell Uqcrc1 (complex III) and Atp5a1 (ATP synthase) gene expression. In addition, both HIIT and high concentrations of lactate significantly elevated ATP levels. These results suggested that HIIT probably acts through lactate to increase hippocampal mitochondrial OXPHOS and energy generation efficiency to meet high brain energy demands, such as BDNF production. Then, the expression of BDNF may further contribute to the beneficial effects of exercise on synaptic plasticity (Raefsky and Mattson, 2017). However, the question of how lactate promotes mitochondrial function to induce BDNF expression needs to be solved.

As described in previous studies, 2 weeks of intraperitoneal administration of 18 mM lactate can mimic the effect of 7 weeks of exercise on improving PRC expression and increasing mtDNA copy number in the mice brain (Lezi et al., 2013). Furthermore, 20 mM lactate was proven to stimulate the protein expression of PGC-1α in mouse neurons (El Hayek et al., 2019) and regulate mitochondrial biosynthesis signals in L6 cells (Hashimoto et al., 2007). Hence, lactate is the pivotal element for mitochondrial biogenesis and thus mitochondrial function improvement. Moreover, physical exercise has been confirmed to increase the expression of proteins involved in mitochondrial fusion and biogenesis, decrease the expression of the mitochondrial fission-related protein DRP1, and simultaneously alter mitophagy markers. All of the changes in the mitochondrial quality control system in the brain are beneficial for increasing mitochondrial function and enhancing brain function (Marques-Aleixo et al., 2015; Luo et al., 2017). Considering the effect that the influence of exercise on mitochondrial function is guaranteed by the mitochondrial quality control system, we speculate that lactate may also participate in mediating this system to maintain the mitochondrial function during HIIT. The mitochondrial quality control system regulates bioenergetic efficiency and energy expenditure. The system includes mitochondrial fusion, fission, mitophagy, and mitochondrial biogenesis. Normally, mitochondria are dynamic organelles that continually fuse and divide, which determines mitochondrial morphology and allows their immediate adaptation to energetic needs. Mitochondrial fusion can repair dysfunctional mitochondria by mixing the contents with healthy mitochondria to regulate MFN1, MFN2, and OPA1 (Rovira-Llopis et al., 2017). Studies show that acute or chronic exercise increases energy demand and significantly promotes MFN2 and OPA1 protein expression. Then, the mitochondria show obvious fusion tendencies. With the enhancement of fusion, the levels of OXPHOS and ATP complexes are significantly elevated, resulting in an increase in ATP levels (Yao et al., 2019). Mitochondrial fission segregates badly damaged mitochondria, which is regulated by DRP1 and mitochondrial FIS1 (Cai and Tammineni, 2016). Under the stimulation of various factors, DRP1 is recruited into the mitochondria and undergoes oligomerization. Several DRP1 molecules closely surround the mitochondria to form a ring structure, which hydrolyzes GTP depending on the activity of its GTPase, causing the inner and outer membranes of mitochondria to break and leading to mitochondrial fission (Ji et al., 2015). Reduced fusion and increased mitochondrial

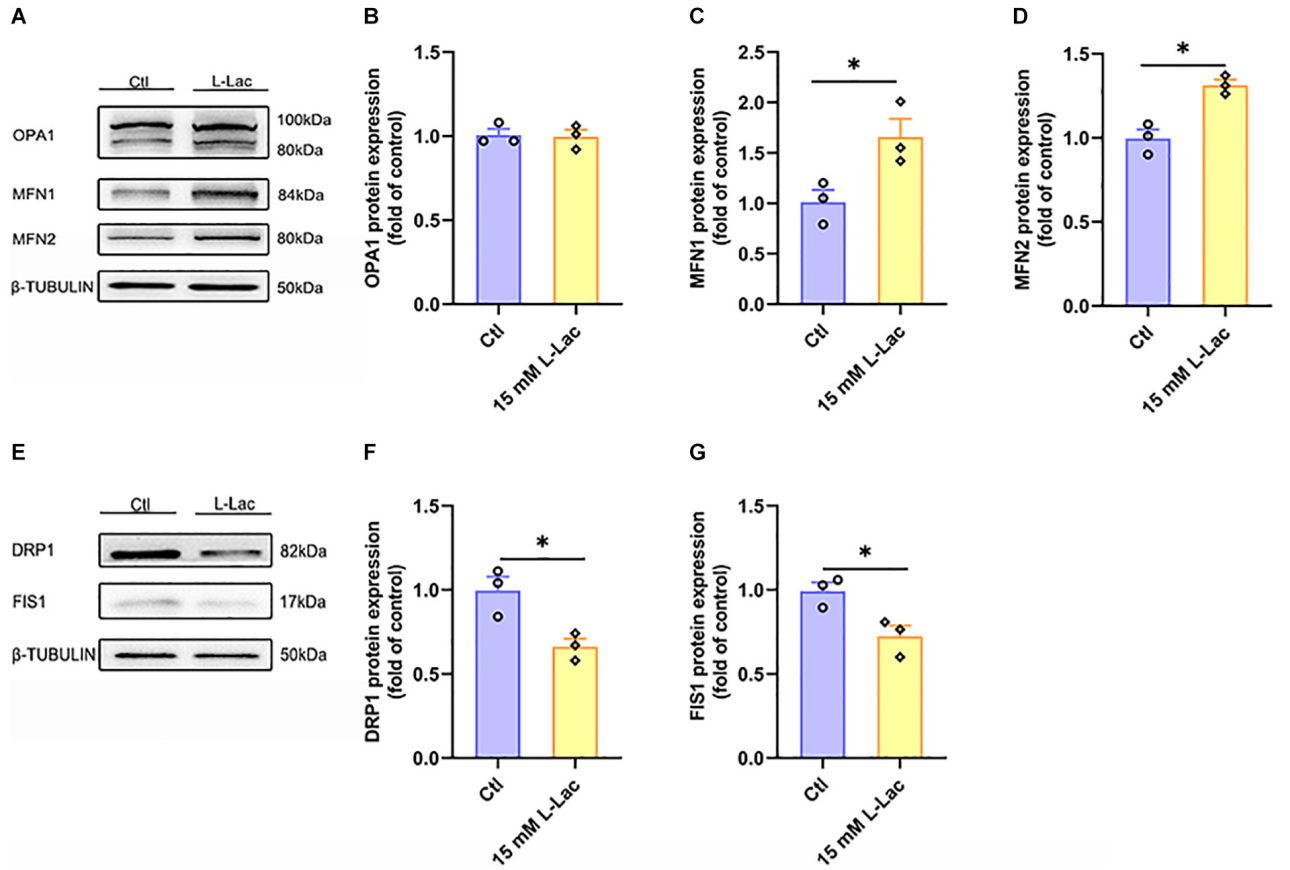

FIGURE 9 | Lactate directly regulated mitochondrial fusion and fission in primary hippocampal cells. The Optic atrophy (OPA1), Mitofusin 1 (MFN1), and Mitofusin 2 (MFN2) were determined by WB to observe the effect of lactate on the mitochondrial fusion in primary hippocampal cells. The Dynamin-related protein1 (DRP1) and mitochondrial fission 1 protein (FIS1) were also determined by WB to observe the effect of lactate on the mitochondrial fusion in primary hippocampal cells. **(A,E)** Representative WB image. **(B)** OPA1 protein expression. **(C)** MFN1 protein expression. **(D)** MFN2 protein expression. **(F)** DRP1 protein expression. **(G)** FIS1 protein expression. TUBULIN as the loading control. $N = 3$ in three independent experiments. $^*P \leq 0.05$ as compared with the Ctl group by non-parametric tests. Values are expressed as mean ± standard error of the mean.

fission may be the primary causes of mitochondrial dysfunction and neuronal damage. In this study, we found that both HIIT and high concentrations of lactate upregulated the protein expression of OPA1, MFN1, and MFN2 to promote mitochondrial fusion but significantly downregulated the protein expression of DRP1 and FIS1 to inhibit mitochondrial fission. The results demonstrated that there was a very high energy requirement in the hippocampus in HIIT, and the significant increase in OXPHOS levels was also the result of enhanced mitochondrial fusion. More importantly, the results verified that lactate may participate in regulating HIIT-triggered hippocampal mitochondrial fusion and fission.

In addition, mitophagy plays a critical role in maintaining mitochondrial quality by degrading aging, damaged, or dysfunctional mitochondria (Tang et al., 2016). Damage to the system that regulates mitophagy can lead to the accumulation of dysfunctional mitochondria, which is a characteristic of aging-related diseases such as Alzheimer's disease and Parkinson's disease. Among the multiple mechanisms by which mitochondria are targeted for degradation at the autophagosome, the best understood and the classical pathway is PINK1-PARKIN-dependent mitophagy. In the presence of damaged mitochondria, PINK1 accumulates on the outer mitochondrial membrane and selectively recruits cytosolic PARKIN, known as a cytosolic E3-ubiquitin ligase, to mitochondria (Chen and Dorn, 2013). Then, PARKIN ubiquitinates the depolarized mitochondria and binds to LC3 to promote the assembly of the autophagic machinery and eliminate damaged mitochondria (Lee et al., 2010). Furthermore, P62 is recruited to mitochondria in a PARKIN- and depolarization-dependent manner. Knockdown of P62 substantially inhibits mitophagy. However, one study noted that P62 recruitment to mitochondria may also be insufficient for mitophagy (Narendra et al., 2010). Anyway, PINK1 and PARKIN, act as the mitochondrial gatekeepers, can sense healthy versus unhealthy mitochondria and can regulate mitochondrial quality control pathways (Leites and Morais, 2018). However, the effect of HIIT or lactate on hippocampal mitophagy remains unknown. Our results showed that both HIIT and high concentrations of lactate had almost no effect on hippocampal mitophagy; namely, HIIT did not damage hippocampal mitochondria, which also

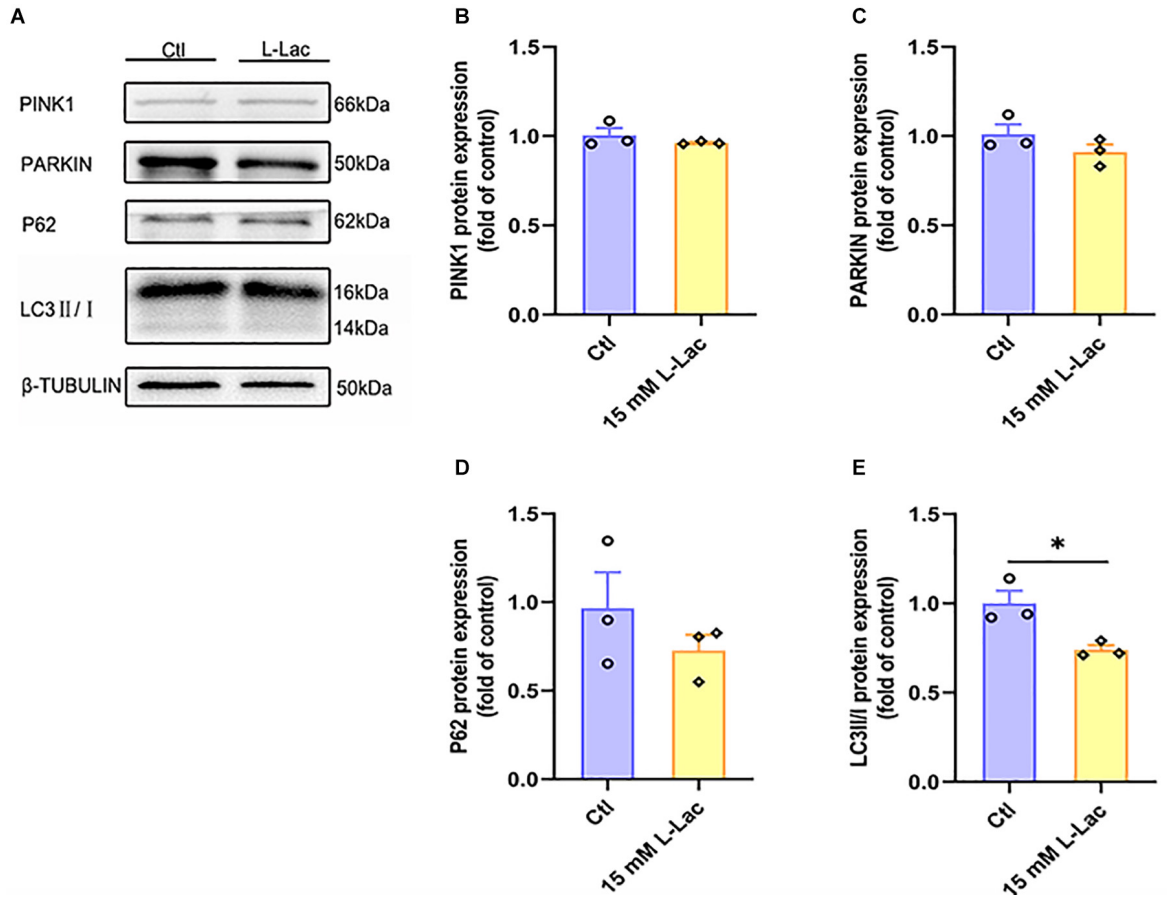

FIGURE 10 | Lactate weakly influenced mitophagy in primary hippocampal cells. The PTEN-induced putative kinase 1 (PINK1), PARKIN, Sequestosome-1 (P62), and microtubule-associated protein light chain3 (LC3) were determined by WB to observe the effect of lactate on the mitophagy in primary hippocampal cells. **(A)** Representative WB image. **(B)** PINK1 protein expression. **(C)** PARKIN protein expression. **(D)** P62 protein expression. **(E)** LC3 protein expression. TUBULIN as the loading control. $N = 3$ in three independent experiments. $^*P \leq 0.05$ as compared with the Ctl group by non-parametric tests. Values are expressed as mean ± standard error of the mean.

suggested that HIIT is safer for improving cognitive function and brain health.

Mitochondrial biosynthesis is a dynamic process in which new mitochondria form to maintain and restore mitochondrial structure, quantity and function under the conditions of increased energy demand. PGC-1α is considered as the master regulator of mitochondrial biogenesis because it interacts with two key nuclear transcription factors, NRF1 and NRF2, and it increases the expression levels and activities of NRF1 and NRF2 through protein-protein interactions. NRF1 and NRF2 activate mitochondrial TFAM and bind to promoter regions of nuclear genes encoding subunits of the five complexes in the mitochondrial electron transport chain, thereby increasing mtDNA replication (namely, mtDNA copy number) for mitochondrial biogenesis (Sharma et al., 2013). The mitochondrion is the only organelle that contains its own DNA (mtDNA) outside the nucleus, and mtDNA copy number reflects the abundance of mitochondria within a cell and the cellular energy status of tissues. Generally, in mitochondrial diseases, a mutation in the mtDNA leads to a loss of functionality of the OXPHOS, and that can lead to a depletion of ATP, which can, in turn, induce further mtDNA mutations. One study showed that high-intensity exercise, a kind of supra-lactate threshold exercise, increased hippocampal mtDNA copy number and activated a partial mitochondrial biogenesis in aged mice, which may benefit protection against decreased cognitive function in the aging brain (Lezi et al., 2014). Here, we also found that both HIIT and high concentrations of lactate treatment could promote hippocampal PGC-1α and NRF2 protein expression and increase mtDNA copy number, which demonstrated that HIIT could facilitate hippocampal mitochondrial biogenesis through lactate to accommodate high energy requirements. Additionally, we found an interesting phenomenon in which the protein expression of NRF1 only had an increasing tendency but no significant difference following HIIT or lactate treatment. These *in vitro* results were consistent with previous research conclusions (Hashimoto et al., 2007). As described above, our results elucidated that lactate participated in mediating the effect of HIIT on mitochondrial biogenesis in the mouse hippocampus by activating PGC-1α- NRF2 signals.

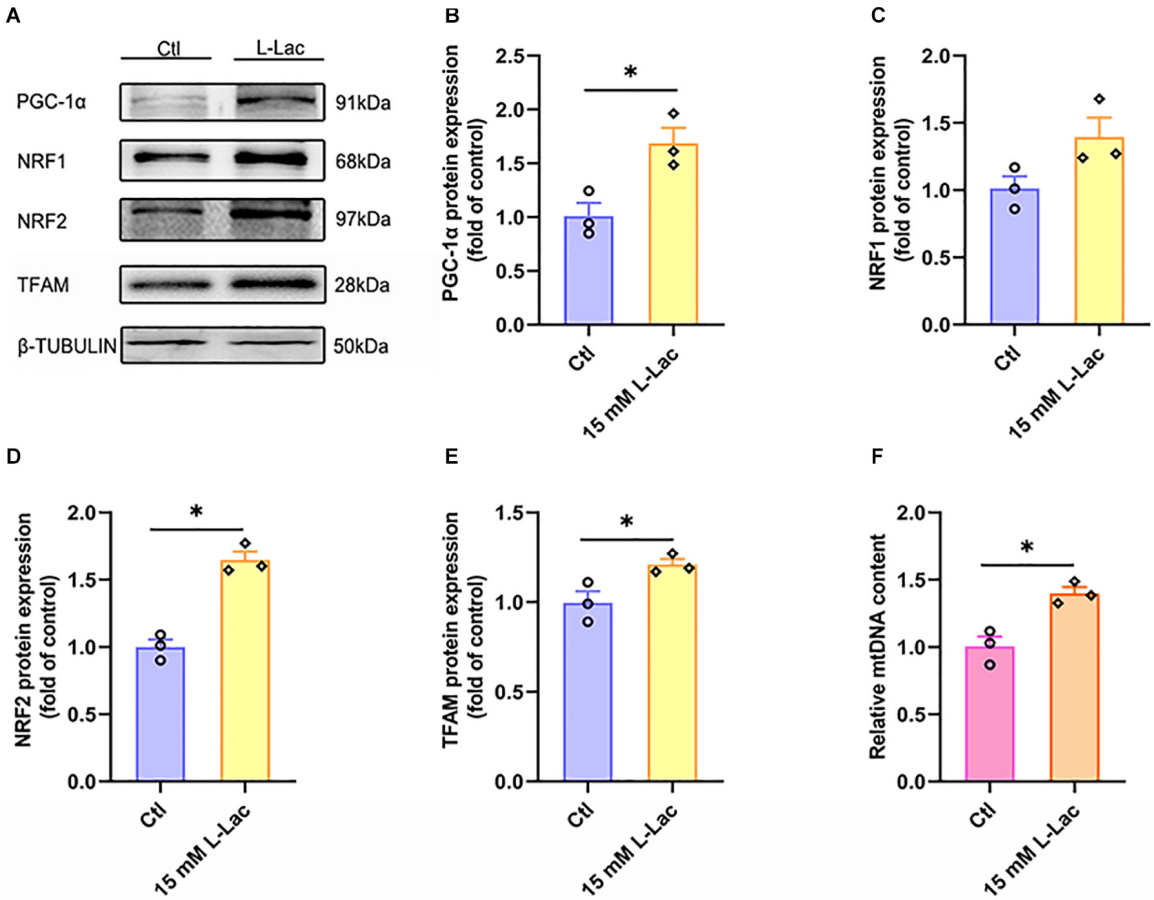

FIGURE 11 | Lactate enhanced mitochondrial biogenesis signal and increased mitochondria DNA copy number in primary hippocampal cells. The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factors 1 (NRF1), nuclear respiratory factors 1 (NRF2), and transcription factor A (TFAM) were determined by WB to observe the effect of lactate on mitochondrial biogenesis signals in primary hippocampal cells. The mt-Cytb level was determined by RT-PCR to evaluate mitochondria DNA copy number after the L-lactate treatment in primary hippocampal cells. **(A)** Representative WB image. **(B)** PGC-1α protein expression. **(C)** NRF1 protein expression. **(D)** NRF2 protein expression. **(E)** TFAM protein expression. **(F)** mt-Cytb level. TUBULIN and Cycs as the loading control. $N = 3$ in three independent experiments. $^*P \leq 0.05$ as compared with the Ctl group by non-parametric tests. Values are expressed as mean ± standard error of the mean.

In this study, we found 6-week HIIT through elevating hippocampal lactate levels could regulate hippocampal mitochondrial quality control system to improve mitochondrial function, which may then promote BDNF expression. Notably, the potential mechanism is unknown how the increased hippocampal lactate regulates mitochondrial fusion, fission, and biosynthesis during HIIT. Nonetheless, our results suggest that HIIT is beneficial for hippocampal mitochondrial function and BDNF expression, and lactate plays the vital signaling role. However, it should be noted that the mice hippocampal cell culture experiment with 15 mM lactate treatment for 3 h is not enough to represent what has happened actually in peripheral and central physiological changes of lactate induced by HIIT. Different mode of HIIT and the characteristics of rapid metabolism of lactate *in vivo* will inevitably make the blood and brain lactate level flexible. Therefore, it is worth considering whether lactate itself could serve as the effective exercise mimetic to exert the effect of enhancing brain mitochondrial function. Although lactate is not completely responsible for all exercise effect, it does regulate the mitochondrial quality control system in hippocampal cells. It is of significance to investigate the link of lactate, exercise, and brain mitochondrial function because that may help us understand how exercise benefits brain health.

CONCLUSION

This is the first study to demonstrate that 6-week HIIT can enhance mice hippocampal mitochondrial fusion and biogenesis and inhibit mitochondrial fission to lift mitochondrial energy production function, which may promote the BDNF expression. This process is mainly attributed to the novel signal role of lactate. However, there are some limits in this study. Firstly, the energy substrate role of lactate in mitochondrial function cannot be excluded because lactate converts into pyruvate for mitochondrial OXPHOS and ATP production. In the future,

we will focus on how this dual role of lactate influences mitochondrial function in the brain. Secondly, our results cannot show an obvious evidence that HIIT-mediated mitochondrial quality control system changes would directly contribute to the expression of BDNF.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Science Research at Shanghai.

AUTHOR CONTRIBUTIONS

JH completed experiment and drafted the manuscript. MC assisted with drafting manuscript and drawing the graph. QS, ZL, YF, BL, and XX assisted with keeping animals and partial experiment. SL conceptualized the article and revised the final version. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.629914/full#supplementary-material

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frontiers in Physiology

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Hydroxycarboxylic Acid Receptor 1 and Neuroprotection in a Mouse Model of Cerebral Ischemia-Reperfusion

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Lactate is an intriguing molecule with emerging physiological roles in the brain. It has beneficial effects in animal models of acute brain injuries and traumatic brain injury or subarachnoid hemorrhage patients. However, the mechanism by which lactate provides protection is unclear. While there is evidence of a metabolic effect of lactate providing energy to deprived neurons, it can also activate the hydroxycarboxylic acid receptor 1 (HCAR1), a Gi-coupled protein receptor that modulates neuronal firing rates. After cerebral hypoxia-ischemia, endogenously produced brain lactate is largely increased, and the exogenous administration of more lactate can decrease lesion size and ameliorate the neurological outcome. To test whether HCAR1 plays a role in lactate-induced neuroprotection, we injected the agonists 3-chloro-5-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid into mice subjected to 30-min middle cerebral artery occlusion. The *in vivo* administration of HCAR1 agonists at reperfusion did not appear to exert any relevant protective effect as seen with lactate administration. Our results suggest that the protective effects of lactate after hypoxia-ischemia come rather from the metabolic effects of lactate than its signaling through HCAR1.

Keywords: metabolism, middle cerebral artery occlusion, ischemia, neuroprotection, lactate, hydroxycarboxylic acid receptor 1

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INTRODUCTION

Lactate, a presumed trivial metabolite of glycolysis, has gained a lot of interest in neuroscience in recent years. On the one hand, lactate is a preferred energy substrate of neurons and can be provided to neurons by astrocytes (Pellerin and Magistretti, 1994); on the other hand, lactate acts as a volume transmitter and mediator of metabolic information in the neurovascular unit [For review: (Bergersen and Gjedde, 2012)]. Lactate plays a critical physiological role in long-term memory formation and plasticity (Suzuki et al., 2011; Yang et al., 2014) and has been shown to have beneficial effects in animal models of acute brain injuries, including ischemic stroke (Rice et al., 2002; Berthet et al., 2009, 2012; Castillo et al., 2015; Zhou et al., 2018; Buscemi et al., 2020), and in patients suffering from traumatic brain injury or subarachnoid

hemorrhage (Bouzat et al., 2014; Carteron et al., 2018). However, the mechanism by which lactate provides neuroprotection is not clear.

While there is evidence that lactate has a beneficial metabolic effect providing energy to deprived neurons (Schurr et al., 1997, 1999; Roumes et al., 2020), it can also activate the hydroxycarboxylic acid receptor 1 (HCAR1), a Gi-coupled protein receptor (Cai et al., 2008; Ahmed et al., 2009) that modulates neuronal firing rates (Bozzo et al., 2013; Herrera-Lopez and Galvan, 2018; de Castro Abrantes et al., 2019). After cerebral hypoxia-ischemia, endogenously produced brain lactate is largely increased (Harada et al., 1992; Lei et al., 2009; Alf et al., 2012; Hyacinthe et al., 2020), and the exogenous administration of more lactate, systemically or directly into the brain, can decrease the lesion size and ameliorate the neurological outcome (Berthet et al., 2009, 2012; Castillo et al., 2015; Buscemi et al., 2020; Roumes et al., 2020). As lactate could be used as a metabolic substrate and/or trigger a signaling response after binding to its receptor, a dual mechanism of action was envisaged for lactate neuroprotection. Indeed, there is *in vivo* evidence that exogenous lactate administered after ischemia can quickly reach the brain and is converted into pyruvate and further oxidized (Hyacinthe et al., 2020; Roumes et al., 2020). Also, we have *in vivo* and *in vitro* evidence that D-lactate exerts neuroprotection, and *in vitro* evidence that the activation of the Gi-coupled lactate receptor without any direct effect on metabolism can have a neuroprotective effect after oxygen and glucose deprivation (OGD; Castillo et al., 2015).

While lactate receptor agonists have been tested using several *in vitro* paradigms (Dvorak et al., 2012; Liu et al., 2012; Bozzo et al., 2013; Lauritzen et al., 2014; Castillo et al., 2015; Herrera-Lopez and Galvan, 2018; Vardjan et al., 2018; de Castro Abrantes et al., 2019; Vohra et al., 2019), their effects have been only recently tested in the central nervous system with unclear results (Lev-Vachnish et al., 2019; Scavuzzo et al., 2020). To further investigate in this direction and shed some light on a presumable dual protective effect of lactate, we tested if HCAR1 stimulation could provide neuroprotection when administered *in vivo* in a mouse model of transient hypoxia-ischemia. With this aim, we evaluated the effect on the ischemic lesion size and the neurological outcome of two different compounds, the synthetic 3-chloro-5-hydroxybenzoic acid (CHBA) and 3,5-dihydroxybenzoic acid (DHBA), a metabolic product of the β-oxidation of alkylresorcinols found in cereals such as rye and wheat (Ross et al., 2004), both of them considered HCAR1 full agonists.

MATERIALS AND METHODS

Transient Middle Cerebral Artery Occlusion Model

For our *in vivo* experiments, we used a well-established mouse transient middle cerebral artery occlusion (MCAO) model of ischemic stroke with reperfusion (Longa et al., 1989) on 8–10 weeks male C57BL/6 J mice (Charles River, France). The veterinary authorities of Canton Vaud according to the Federal guidelines of the Swiss Veterinary Office approved the experimental and surgical procedures. Mice had *ad libitum* access to food and water and were housed under standard conditions, except for postsurgical recovery, when they were kept overnight in their home cages inside a temperature-controlled incubator at 28°C. Ischemia was induced by inserting a silicone-coated suture (Doccol) through the left common carotid artery into the internal carotid artery and advancing it into the arterial circle to occlude the origin of the middle cerebral artery. After 30 min, the coated suture was withdrawn and the cerebral blood flow (CBF) restored. The whole procedure was done under laser Doppler CBF control, maintaining the rectal temperature at 37 ± 0.5°C. The surgery was considered successful if the CBF during occlusion reached below 20% of the initial value and reperfusion reached at least 50% of the initial value within 10 min of filament removal. Mice received subcutaneous injections of buprenorphine (0.025 mg/kg) pre- and post-surgery for analgesia.

Lesion Volume Measurement

Lesion volumes were measured from cresyl violet-stained serial coronal (20-μm thick and 720-μm apart) cryostat sections of liquid nitrogen vapor-frozen tissue obtained 48 h after MCAO surgery (Buscemi et al., 2020; **Figure 1**). Images were taken with a Nikon SMZ25 stereomicroscope and were measured while blinded to the treatment using ImageJ. The direct infarct size was calculated as the sum of the infarcted areas on each section multiplied by the spacing distance between sections. The indirect infarct size was calculated as the subtraction of the healthy ipsilateral volume from the contralateral volume. The percentage of cerebral hemisphere swelling was calculated as the difference between ipsilateral and contralateral volumes divided by the total brain volume.

Functional Outcome Assessment

We assessed the functional outcome of mice by neurological score evaluation, Rotarod test, and/or wire-hanging test (Blochet et al., 2020; Buscemi et al., 2020). We performed the assessment and analysis of the behavioral outcomes blinded to the treatment.

We evaluated the neurological deficit on all mice immediately after surgery and at 24 h and 48 h after MCAO and graded for severity using the following scale: 0 for no observable deficit, 1 for failure to extend the forepaw, 1.5 for intermittent circling, 2 for persistent circling, and 3 for loss of circling or righting reflex.

A set of mice were subjected to the Rotarod test, where they were placed on an accelerating (4 to 40 rpm) rotating cylinder (Ugo Basile) and left to run for a maximum of 900 s. We trained mice for three consecutive days before surgery and tested them 24 h and 48 h after MCAO. The longest latency to fall from the rod over three trials was scored for each time point.

We subjected all mice to the wire-hanging test, where they were suspended on a single wire stretched between two posts above a soft ground and allowed to escape toward the posts. We trained mice 1 day before intervention and scored for escaping and/or falling events at 24 h and 48 h after MCAO.

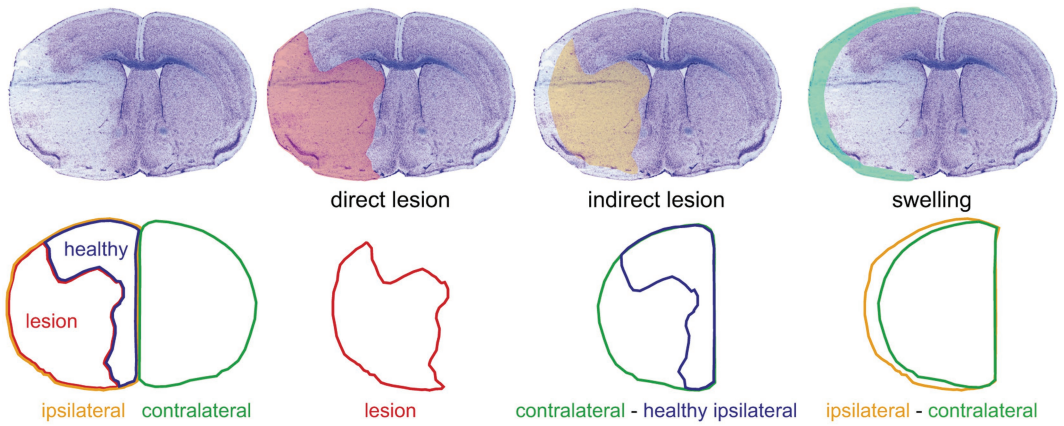

FIGURE 1 | Evaluation of the ischemic damage. The upper row shows (from left to right) a representative image of a cresyl violet-stained coronal section of a 48 h post-middle cerebral artery occlusion mouse brain and the overlays on the same section of the corresponding direct lesion (red shading), indirect lesion (yellow shading), and swelling (light green shading). The lower row shows the outlines of the different areas considered [ipsilateral hemisphere area (yellow outline), lesion area (red outline), healthy ipsilateral area (blue outline), and contralateral hemisphere area (green outline)] and the operations used to measure the damage illustrated in the corresponding upper row. This procedure was done on a set of 12 serial sections per brain to estimate the lesion and swelling volumes (see "Materials and Methods" section).

The test lasted for a maximum of 180 s on the wire or a maximum of 10 falls. We evaluated the overall performance as (from better to worse) only escape, neither escape nor fall, escape or fall, and only fall. We also evaluated the number of falls, the number of escapes, and the maximal latency time to fall from the wire. We used The Observer software (Noldus) for data extraction.

Lactate Receptor Agonist Treatments

Mice with successful reperfusion were injected with either the HCAR1 agonists (CHBA or DHBA) or with vehicle (PBS). Only a single intravenous (IV) or intracerebroventricular (ICV) injection of the agonist or vehicle was given, 15–20 min after filament removal. We administered the treatments in a randomized fashion by an investigator blinded to the treatment. The doses were chosen as explained in the results.

IV Injections

For the pilot dose-response of CHBA IV injection, the agonist was used at 0.1 μmol/g or 0.025 μmol/g. We used 25 mice for this experiment; two mice were discarded due to insufficient reperfusion and six mice due to unsuccessful IV injection. An experiment with a larger number of animals per group was done with an IV injection of 0.1 μmol/g CHBA or vehicle. For this experiment, 30 mice were used; six mice were discarded due to insufficient reperfusion and two mice due to unsuccessful IV injection.

ICV Injections

For the ICV administration of the CHBA experiment, 2 μl of 1 mmol/L CHBA (total dose 2 nmol per mouse) or PBS were delivered into the left ventricle [0.9 mm laterally, 0.1 mm posteriorly, and 3.1 mm deep from bregma (Hirt et al., 2004)]. We used 16 mice for this experiment; one mouse was discarded due to insufficient reperfusion and one mouse injected with PBS had to be sacrificed before the end of the experiment. For the ICV administration of DHBA experiment, 2 μl of 100 mmol/L DHBA (total dose 0.2 μmol per mouse) or PBS were delivered into the left ventricle (see above). We used 18 mice for this experiment; one mouse was discarded due to insufficient reperfusion, one mouse injected with PBS had to be sacrificed before the end of the experiment, and two more mice were discarded due to failed injection. One of the animals had a swollen hind limb (unrelated to the MCAO) and was not considered fit for the behavioral test.

Statistical Analysis

We compared continuous variables following Gaussian distributions with one-way ANOVA with Tukey's *post-hoc* test (multiple groups) or with unpaired two-tailed Student's *t*-test (two groups). We compared non-Gaussian distributions, with the Kruskal–Wallis test. For analysis across time, we used two-way ANOVA. Statistical tests were run on GraphPad Prism 6.0. On the box-and-whisker plots, the line shows the median and the whiskers correspond to the maximum and minimum values. On the floating bar plots, the line shows the mean value. We considered significance as $p < 0.05$.

RESULTS

To better understand the role of the lactate receptor HCAR1 in lactate-induced neuroprotection, we started by testing the potent agonist CHBA. The apparent EC_{50} of CHBA acting on HCAR1 calculated from *in vitro* data is 0.02 mM (Dvorak et al., 2012; de Castro Abrantes et al., 2019), 100–250 times lower than the effective lactate concentrations used *in vitro* (Cai et al., 2008; Liu et al., 2009). To the best of our knowledge, there is no published information on the pharmacokinetics

(bioavailability, permeability, or transport) of any of the HCAR1 agonists. We chose therefore two CHBA doses to test based on the only report showing *in vivo* effects of this agonist after intravenous administration in mice, of 0.15 μmol/g (Wallenius et al., 2017). In an exploratory experiment, we injected intravenously 0.1 μmol/g CHBA, 0.025 μmol/g CHBA or vehicle to mice subjected to 30-min MCAO, shortly after reperfusion (**Figure 2**).

We did not observe any significant change in lesion size or behavioral outcome with any of the two tested doses. However, despite the low number of animals used, there appeared to be a trend for a beneficial effect on lesion size and early outcome at the higher dose of CHBA, whereas the lower dose was suggestive of larger lesion sizes. Then, we performed a second experiment using 0.1 μmol/g CHBA on a larger number of animals (**Figure 3**). With this larger sample size, we did not observe any significant effect of the agonist treatment on lesion size or neurological outcome.

Several studies have shown *in vitro* effects of CHBA on cells of the central nervous system (namely, in neuronal and astrocytic cultures; Vardjan et al., 2018; de Castro Abrantes et al., 2019), but to date the only reported effects of CHBA *in vivo* come from experiments analyzing peripheral effects (Dvorak et al., 2012; Wallenius et al., 2017). We could not discard that the weak effects observed in our experiments might be related to the compound's default in crossing the blood-brain barrier; hence, we decided to perform an experiment with ICV administration of the agonist. As the effective dose of CHBA *in vitro* is 100 times lower than the effective dose of L-lactate, and the drug is delivered directly to the brain, we chose to inject 1 mmol/L CHBA intracerebroventricularly, a concentration 100 times lower than that of L-lactate (Berthet et al., 2009; **Figure 4**). Similar to the experiments with IV administration, the ICV route did not show any significant effect on lesion size or neurological outcome.

Another HCAR1 agonist, the less potent DHBA, which has an apparent EC_{50} of 0.15 mM (Liu et al., 2012), has been shown to exert effects similar to lactate in cultured neurons and acute hippocampal slices (Bozzo et al., 2013; Herrera-Lopez and Galvan, 2018). Previous work from our laboratory showed that DHBA elicited neuroprotection in hippocampal organotypic slices subjected to OGD when used at the same dose as L-lactate (Castillo et al., 2015). In light of our previous results with CHBA, and to ensure delivery to the brain, we directly tested DHBA in our MCAO model using ICV administration of the agonist at 100 mmol/L, the same concentration used for L-lactate (**Figure 5**). Again, no significant effects were observed on lesion size or neurological outcome. However, of note, the results of the DHBA treatment seem to point toward slightly better post-MCAO outcomes than those obtained using the more potent CHBA.

DISCUSSION

The administration of lactate receptor HCAR1 agonists to mice subjected to a transient ischemic insult did not appear to exert a protective effect in terms of lesion size or neurological outcome. This is in contrast to the effect of lactate administration (Berthet et al., 2009). At first, we considered the possibility that the lack of effect of CHBA, a potent HCAR1 agonist shown to activate the receptor *in vitro*, might have been related to its failure to reach the brain following intravenous administration. However, the absence of clear effects after the ICV administration of both CHBA and DHBA suggests that there might be other reasons.

The most straightforward explanation would be that lactate neuroprotection is mainly due to the nurturing effect offered by this metabolite. In opposition to the previous assumptions that associated elevated lactate with poor outcomes, we could rather think that increased production of lactate is an attempt of the brain to restore the calm after the storm. Then, the initial lactate paradox resolves, as it would not be surprising to observe a benefit when giving more of an endogenously produced good thing. Both endogenous and exogenous lactate could be used by astrocytes and suffering neurons to obtain anaplerotic precursors and to produce ATP (Jourdain et al., 2016; Magistretti and Allaman, 2018). At rest, under stimulation, and after intravenous lactate injection, lactate flows from astrocytes to neurons (Mächler et al., 2016; Zuend et al., 2020). And astrocytes with their end-feet in close contact with the blood vessels and lining the ventricles would most likely be the initial recipients of exogenously delivered lactate, *via* the monocarboxylate transporters (MCTs) MCT1 and MCT4. One of the sources of astrocytic-produced lactate is glycogen. Following astrocytic stimulation, lactate can be produced from internal glycogen stores, released to the extracellular space, and be further taken up by neurons *via* MCT2 to sustain their activity in response to the stimulus (Dinuzzo et al., 2012; Goncalves et al., 2018; Magistretti and Allaman, 2018). The addition of exogenous lactate could thus prevent the depletion of these stores while providing astrocytes with plenty of energy and building blocks to sustain survival and further proliferation. Moreover, this abundance of lactate could be shared with the neurons, allowing them to convert it into pyruvate, which would enter the TCA cycle and generate enough ATP to prevent neuronal swelling due to Na^+/K^+-ATPase failure (Magistretti and Allaman, 2018). This process also generates NADH, which has been proposed to influence NMDA receptor excitability (Jourdain et al., 2018). It could also allow the neurons to spare blood-borne glucose for energy production and drive it to feed the pentose phosphate pathway and provide NADPH that would improve their redox buffering capacity (Herrero-Mendez et al., 2009).

In support of the mainly metabolic effect of lactate, we have shown recently that IV administered lactate is metabolized very rapidly in the ischemic mouse brain. Using magnetic resonance spectroscopy after hyperpolarized [1-^{13}C] lactate administration at therapeutic doses, we observed a peak of ^{13}C-lactate followed within seconds by peaks of ^{13}C-labeled pyruvate and bicarbonate in the brain (Hyacinthe et al., 2020), implying intracellular transport and metabolism of the exogenous lactate. Consistently, and even following intraperitoneal injection,

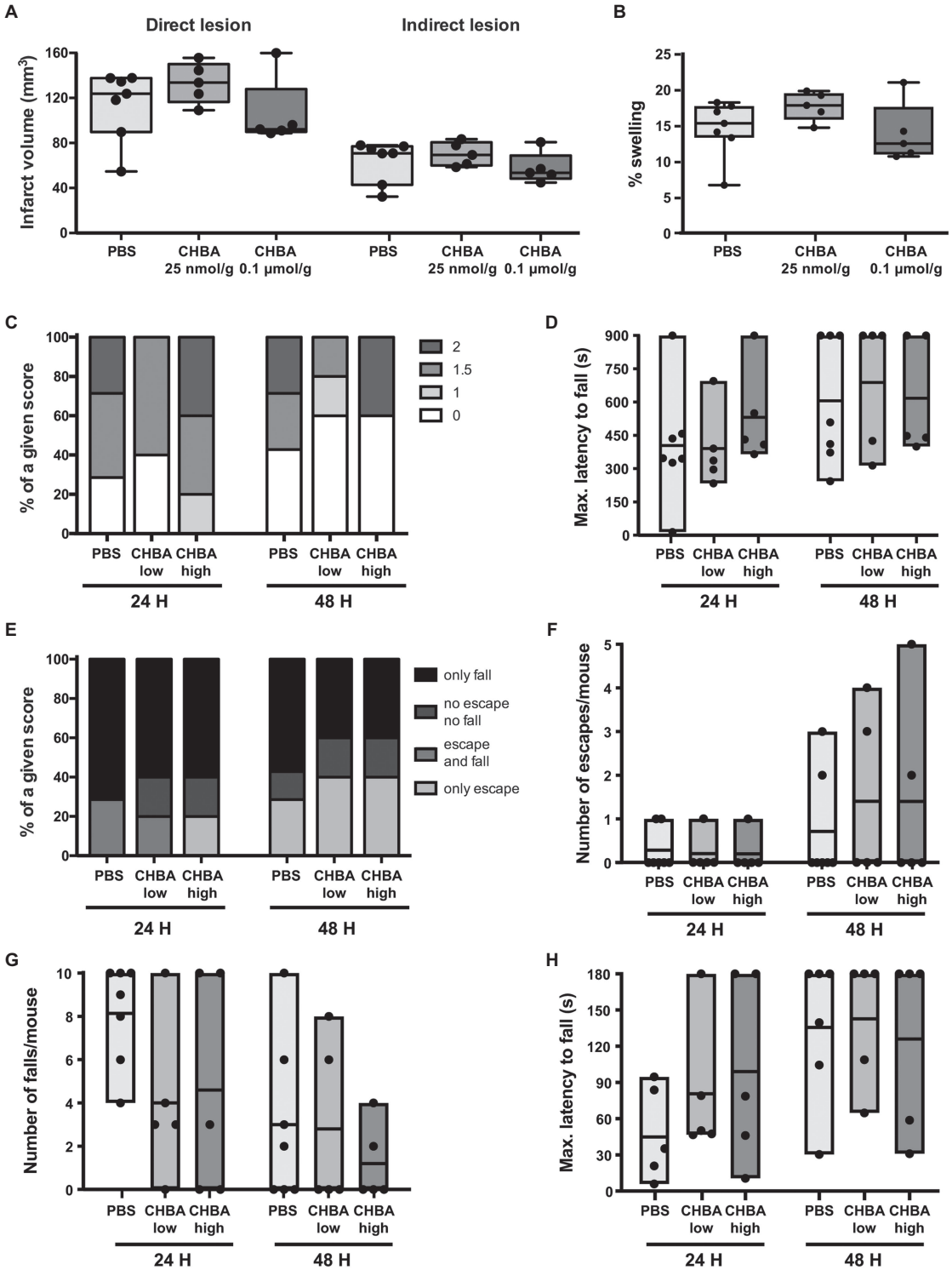

FIGURE 2 | Effect of the intravenous (IV) administration of two different doses of the lactate receptor agonist 3-chloro-5-hydroxybenzoic acid (CHBA) after 30 min MCAO. **(A)** Direct (left) and indirect (right) lesion volumes and **(B)** percentage of swelling from brains of mice that received an IV injection of vehicle (PBS, $n = 7$), a low dose (25 nmol/g, $n = 5$) or a high dose (0.1 µmol/g, $n = 5$) of CHBA at reperfusion. Measurements were done on cresyl violet-stained serial coronal sections of frozen brains from mice sacrificed 48 h after MCAO induction. To evaluate the functional outcome following IV treatment with PBS or CHBA, mice were scored for **(C)** neurological deficit (neuroscores: 0, no deficit; 1, failure to extend right forepaw; 1.5, intermittent circling; 2, circling; and 3, loss of circling or righting reflex), **(D)** performance on Rotarod, **(E)** overall performance on wire-hanging test, **(F)** number of falls from the wire, **(G)** number of times mice escaped from the wire, and **(H)** maximal latency time to fall from the wire. All the behavioral measurements were recorded at 24 h and 48 h after MCAO. Filled dots represent individual animals.

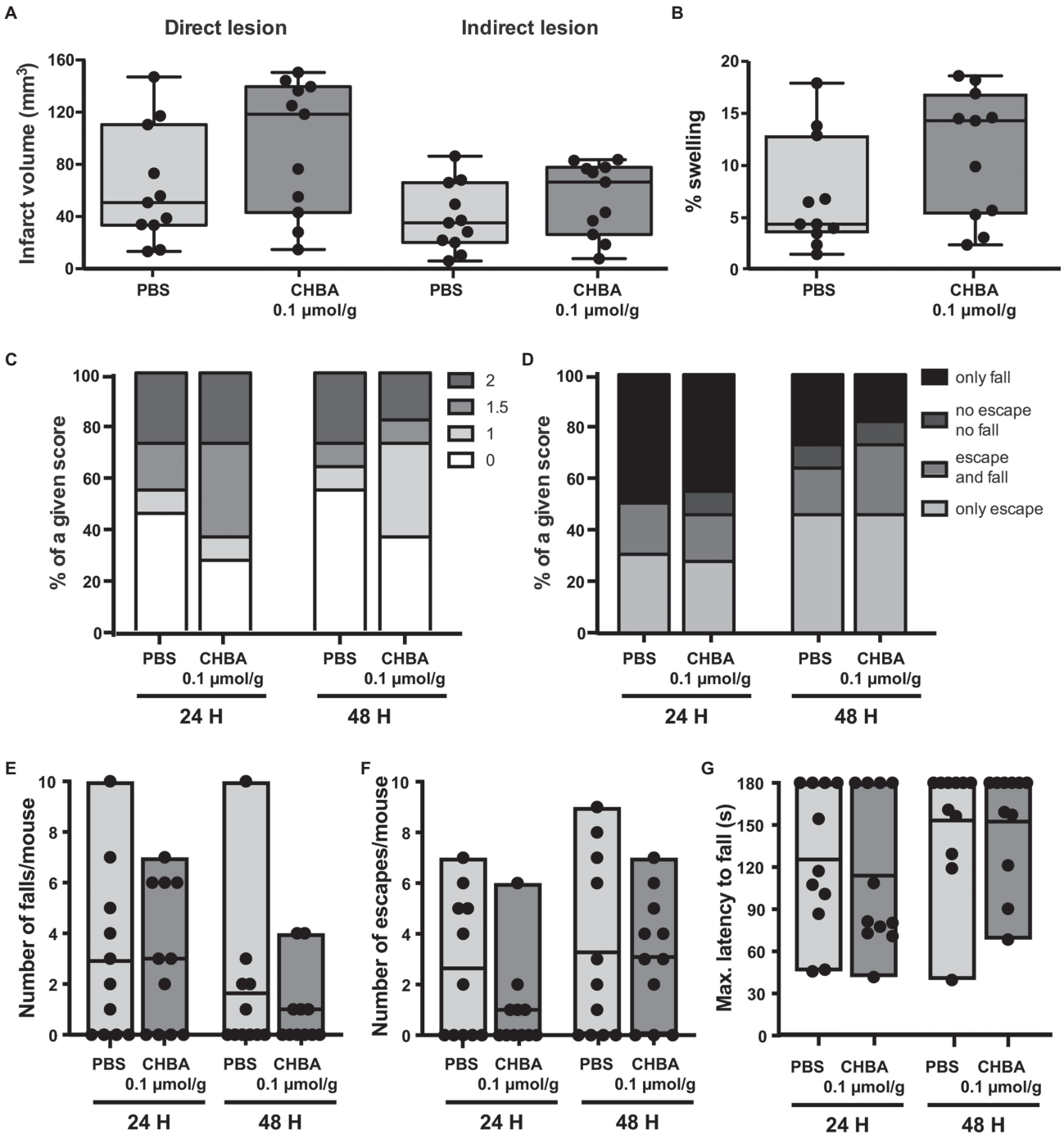

FIGURE 3 | Effect of the intravenous (IV) administration of 0.1 µmol/g of the lactate receptor agonist CHBA after 30 min MCAO. **(A)** Direct (left) and indirect (right) lesion volumes and **(B)** percentage of swelling from brains of mice that received an IV injection of vehicle (PBS, $n = 11$) or 0.1 µmol/g of CHBA ($n = 11$) at reperfusion. Measurements were done on cresyl violet-stained serial coronal sections of frozen brains from mice sacrificed 48 h after MCAO induction. To evaluate the functional outcome following IV treatment with PBS or CHBA, mice were scored for **(C)** neurological deficit (neuroscores: 0, no deficit; 1, failure to extend right forepaw; 1.5, intermittent circling; 2, circling; and 3, loss of circling or righting reflex), **(D)** overall performance on wire-hanging test, **(E)** number of falls from the wire, **(F)** number of times mice escaped from the wire, and **(G)** maximal latency time to fall from the wire. All the behavioral measurements were recorded at 24 h and 48 h after MCAO. Filled dots represent individual animals.

when administered after induction of hypoxia-ischemia in rat pups, [3-^{13}C]-labeled lactate rapidly reached the brain, with label incorporation into several metabolites reflecting its use on the TCA (Roumes et al., 2020). Further stressing the importance of the metabolic effect of lactate, in the same neonatal hypoxia-ischemia model, the inhibition of lactate

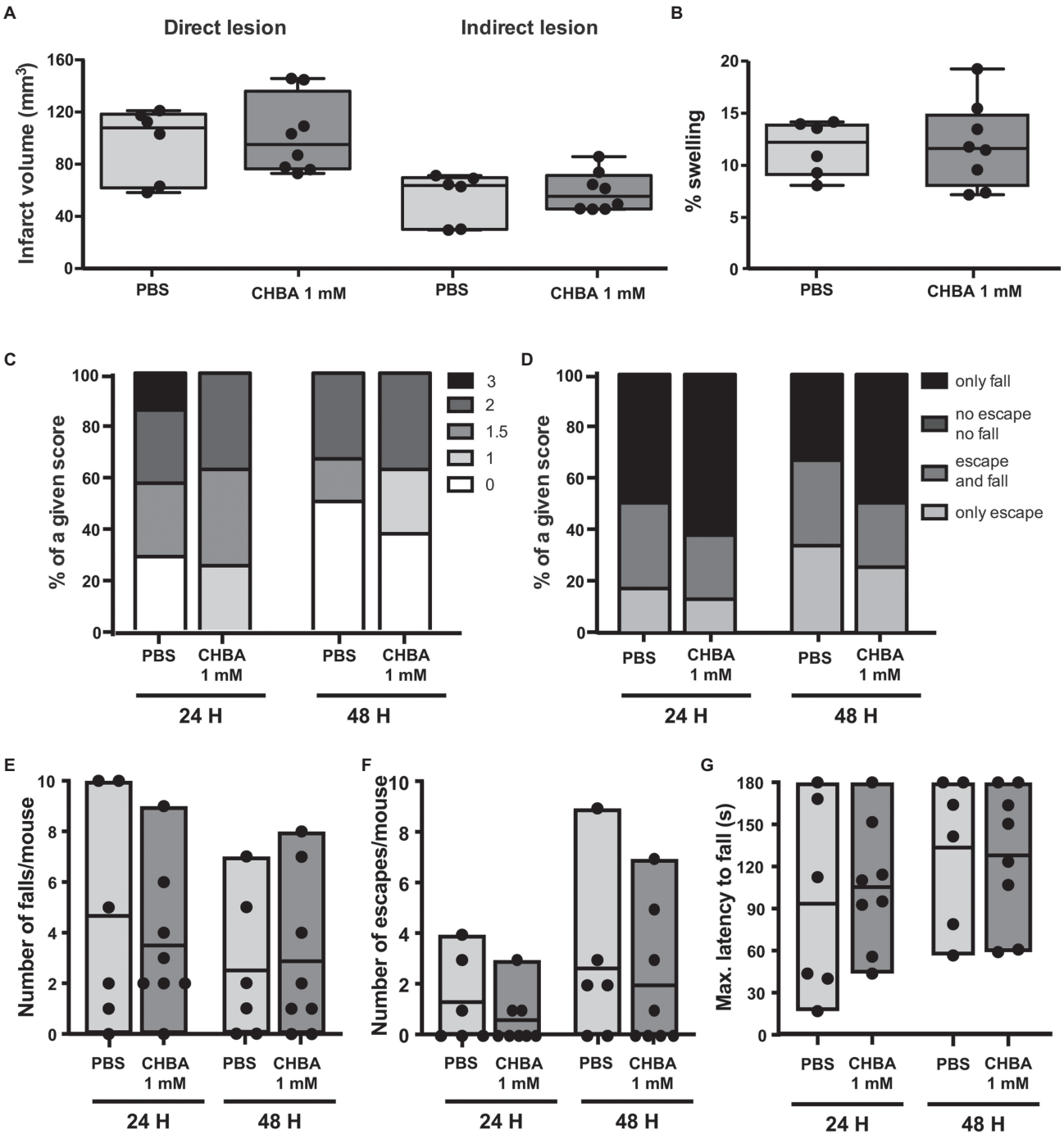

FIGURE 4 | Effect of the intracerebroventricular (ICV) administration of the lactate receptor agonist CHBA after 30 min MCAO. **(A)** Direct (left) and indirect (right) lesion volumes and **(B)** swelling volumes from brains of mice that received a 2 μl ICV injection of vehicle (PBS, $n = 6$) or 1 mmol/L CHBA ($n = 8$) at reperfusion. Measurements were done on cresyl violet-stained serial coronal sections of frozen brains from mice sacrificed 48 h after MCAO induction. To evaluate the functional outcome following ICV treatment with PBS or CHBA, mice were scored for **(C)** neurological deficit (neuroscores: 0, no deficit; 1, failure to extend right forepaw; 1.5, intermittent circling; 2, circling; and 3, loss of circling or righting reflex), **(D)** overall performance on wire-hanging test, **(E)** number of falls from the wire, **(F)** number of times mice escaped from the wire, and **(G)** maximal latency time to fall from the wire. All the behavioral measurements were recorded at 24 h and 48 h after MCAO. Filled dots represent individual animals.

dehydrogenase, the enzyme required to convert lactate into pyruvate, prevented lactate neuroprotection, and there was decreased oxidative damage and a reduction of the cytotoxic edema when administered after the insult (Roumes et al., 2020). Remarkably, the infusion of hypertonic lactate to traumatic brain injury patients increased the brain levels of pyruvate and glucose while leading to a better outcome (Bouzat et al., 2014; Carteron et al., 2018).

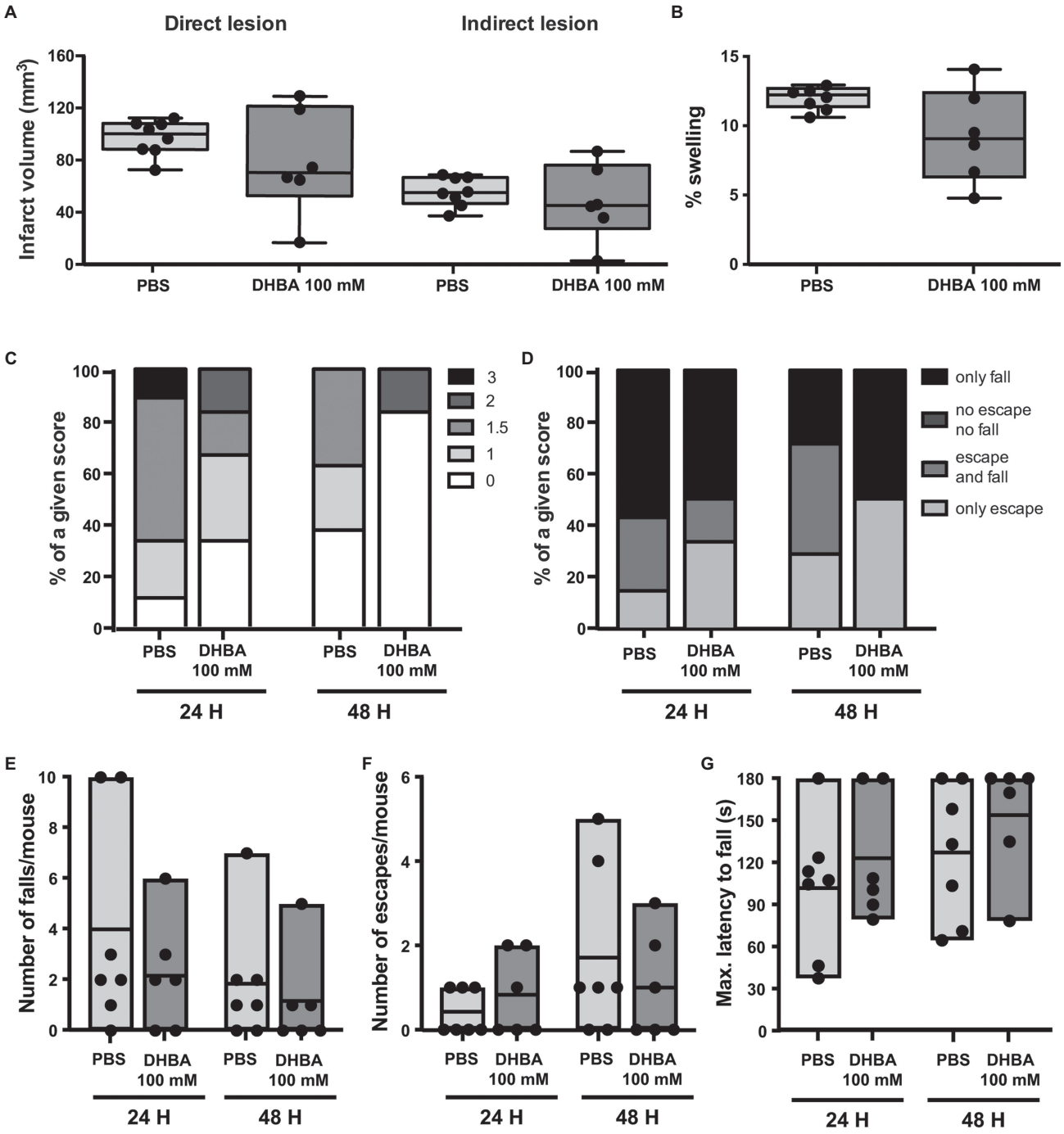

FIGURE 5 | Effect of the ICV administration of the lactate receptor agonist 3,5-dihydroxybenzoic acid (DHBA) after 30 min MCAO. **(A)** Direct (left) and indirect (right) lesion volumes and **(B)** percentage of swelling from brains of mice that received a 2 μl ICV injection of vehicle (PBS, $n = 8$) or 100 mmol/L DHBA ($n = 6$) at reperfusion. Measurements were done on cresyl violet-stained serial coronal sections of frozen brains from mice sacrificed 48 h after MCAO induction. To evaluate the functional outcome following ICV treatment with PBS or DHBA, mice were scored for **(C)** neurological deficit (neuroscores: 0, no deficit; 1, failure to extend right forepaw; 1.5, intermittent circling; 2, circling; and 3, loss of circling or righting reflex), **(D)** overall performance on wire-hanging test, **(E)** number of falls from the wire, **(F)** number of times mice escaped from the wire, and **(G)** maximal latency time to fall from the wire. All the behavioral measurements were recorded at 24 h and 48 h after MCAO. Filled dots represent individual animals. Only seven control mice were taken into account for the wire-hanging test.

D-lactate is a partial agonist of HCAR1 (Cai et al., 2008) that can be metabolized in the brain similarly to L-lactate (Adeva-Andany et al., 2014; Castillo et al., 2015). Similarly to L-lactate, D-lactate has also been shown to protect from the deleterious effects of hypoxia both *in vivo* and *in vitro* (Castillo et al., 2015). However, neurons cannot directly transport

D-lactate due to its weak affinity for MCT2, the main neuronal transporter (Halestrap, 2012). Conversely, endothelial cells and astrocytes, both expressing MCT1, could import and likely metabolize D-lactate. The partial HCAR1 agonist D-lactate, applied at doses similar to those used for neuroprotection was not able to induce changes in the firing frequency of neurons (Herrera-Lopez and Galvan, 2018), although it decreased the neuronal calcium transient frequency (Bozzo et al., 2013). Intriguingly, pyruvate, which is also transported by MCTs and metabolized similarly to lactate, but does not activate HCAR1, induces significant protection against OGD in hippocampal slice cultures but not in the MCAO model (Castillo et al., 2015) and does not protect in the neonatal HI model (Roumes et al., 2020).

Based on the data from *in vitro* experiments, one would expect a protective effect following administration of the lactate receptor agonists, as the extracellular activation of neuronal HCAR1 would slow down neuronal activity (Bozzo et al., 2013; Herrera-Lopez and Galvan, 2018; de Castro Abrantes et al., 2019), exacerbated after the ischemia. Whereas at present we do not have data on the activity of neurons after *in vivo* administration of the agonists, after analyzing the lesion volumes and the behavioral output, we observed that the agonists failed to show any measurable protection from the ischemic damage. Some reports have proposed the existence of other putative lactate receptors, with Gs activity instead of the HCAR1 Gi activity (Tang et al., 2014; Vardjan et al., 2018; D'Adamo et al., 2021). However, the evidence and characterization of these receptors still remain elusive (Mosienko et al., 2018). Others have proposed the interaction of the HCAR1 Giβγ moiety with other GPCRs, and the activation of ERK1/2, PI3K, and Akt pathways following HCAR1 stimulation (Li et al., 2014; de Castro Abrantes et al., 2019; Herrera-López et al., 2020). Additionally, it is not to be excluded that the HCAR1 agonists might interact with receptors other than HCAR1, which might hamper the protection foreseen in relation to HCAR1 activation.

An additional explanation for the lack of protective effect of the agonists in our model, which may help reconcile the evidence provided by *in vivo* and *in vitro* data, could be the interference of the agonists with endogenous lactate. It is well known that the endogenous brain lactate concentration following hypoxia-ischemia shows a fast increase that remains elevated for a long time (Harada et al., 1992; Lei et al., 2009; Alf et al., 2012; Hyacinthe et al., 2020). However, not only hypoxia increases brain lactate concentration. Our experiments were done using isoflurane as an anesthetic. The exposure of mice to isoflurane causes a significant increase in brain lactate concentration (Horn and Klein, 2010; Zuend et al., 2020). Lactate has even been shown to increase in the medium of cells treated with isoflurane (Brabec et al., 1984). The isoflurane-released lactate could be related to the protective effect that it exerts from ischemic insults (Berthet et al., 2009; Burchell et al., 2013; Horn and Klein, 2013), and we had observed that exogenously administrated lactate had a stronger protective effect when using ketamine/xylazine anesthesia compared to isoflurane (Berthet et al., 2009). The presence of isoflurane is an evident difference between our *in vivo* and *in vitro* experiments, which could affect HCAR1 responsiveness to the agonists. Moreover, the isoflurane-driven increase in lactate could lead to relevant changes that could affect lactate transport even before the administration of the drugs. Indeed, animals undergoing sham surgery under isoflurane anesthesia have been shown to experience changes in MCTs, with the increased expression on vessels (Rosafio et al., 2016), suggesting that maybe exposure to isoflurane and the subsequent changes in brain lactate concentrations are sufficient to trigger changes in the distribution of the lactate transporters. The increase in the intracerebral lactate concentration due to the anesthetic, adding up to the lactate surge due to the hypoxia-ischemia could set the stage for an already elevated concentration of lactate. While additional lactate administration still may provide protection, the administration of the more potent HCAR1 agonists added to an already high lactate concentration could bring a different scenario.

The agonists, which are not transported into cells and could thus remain in the interstitial space before being cleared out, might reach high local concentrations near HCAR1 receptors. Although there are no pharmacokinetic data available for the two compounds tested in these experiments, a nutritional study measuring urinary excretion of DHBA after cereal ingestion demonstrated a peak at 10 h, suggesting a long bioavailability (Zhu et al., 2014). At rest, there is a basal tone of HCAR1, with a mild inhibition of adenylate cyclase (AC). Due to the ischemic insult, the steady increase in lactate concentration can reach a level where the receptor is fully activated (Cai et al., 2008; Liu et al., 2009), inhibiting AC and decreasing cAMP levels. The short sustained stimulation of GPCRs like HCAR1 can result in a decreased response over a time frame of minutes due to transducer uncoupling or acute desensitization (Rajagopal and Shenoy, 2018). Further, if the stimulation is sustained over hours, this may result in decreased receptor expression at the plasma membrane due to downregulation or prolonged desensitization (Kelly et al., 2008; Rajagopal and Shenoy, 2018). Indeed, HCAR1 has been shown to internalize after prolonged exposure to agonists *in vitro* (Liu et al., 2009). Therefore, if localized extracellular levels of the agonists (endogenous and/or exogenous) are large enough, a diminished response of HCAR1 could be expected. Supporting this idea, it has been reported that lactate or agonist stimulation of neuronal HCAR1 has opposite effects on the firing frequency depending on their concentrations, with large concentrations giving rise to increased excitability (Herrera-Lopez and Galvan, 2018). Moreover, very high doses of lactate applied after OGD *in vitro* lose the protective effect and can be even toxic (Berthet et al., 2009).

In our MCAO-operated mice, the cumulative presence of a substantial amount of endogenous lactate and potent HCAR1 agonists could have tipped the receptor activation toward hyperexcitability. The overstimulation of the neuronal HCAR1 in the acute post-stroke phase could therefore, lead to a detrimental exacerbation of neuronal activity, instead of slowing it down to balance the effects caused by the excessive extracellular glutamate that accumulates after hypoxia-ischemia. In earlier unpublished experiments, we failed to improve the neurological outcome in mice with short-term repeated lactate

intravenous administration, while single administration was efficient (Berthet et al., 2009; Castillo et al., 2015; Buscemi et al., 2020), suggesting that protracted receptor stimulation was not beneficial. As mentioned before, the protective effects of exogenously administered lactate were stronger when using ketamine/xylazine anesthesia compared to isoflurane. The neuroprotective effects observed when using the agonist DHBA after OGD, an *in vitro* system (Castillo et al., 2015), could be related to the medium change allowing recovery after the OGD, which might have cleared out enough hypoxia-produced lactate to eliminate interference with the agonist and thereby not prevent receptor-mediated protective effects. Although the discrepancy between the effects of DHBA on the *in vitro* vs. *in vivo* models could also be related to the differences between the cellular architecture of organotypic cultures and that of the whole organism, particularly on vascular-related aspects like the presence/absence of circulation, endovascular compartment and blood-brain-barrier. Still, in our *in vivo* experiments, whereas the more potent agonist CHBA tends toward a more deleterious effect, the observed effects after the treatment with the weaker DHBA hint at a slight amelioration of the behavioral outcome.

The present results with the HCAR1 agonists cannot exclude that the beneficial effects of exogenous lactate administration after MCAO could be majorly attributable to a metabolic effect, nor absolutely discard any neuroprotective effect of HCAR1 stimulation. It would be interesting thus to test the effects of exogenous lactate administration to HCAR1 KO mice subjected to MCAO, to use a different anesthetic and/or a delayed administration of the agonists to wild-type mice. Finally, MCT1, MCT2, and MCT4 (Rosafio et al., 2016) lactate transporters show changes in expression and cellular localization after MCAO, which could contribute to lactate's protective mechanism and may be worth a future study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LB, PM and LH contributed to the study conception. LB and LH drafted the manuscript and figures. LB and CB acquired and analyzed the data. All authors contributed to the article and approved the submitted version.

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REVIEW
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Lactate Fluxes and Plasticity of Adipose Tissues: A Redox Perspective

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Lactate, a metabolite produced when the glycolytic flux exceeds mitochondrial oxidative capacities, is now viewed as a critical regulator of metabolism by acting as both a carbon and electron carrier and a signaling molecule between cells and tissues. In recent years, increasing evidence report its key role in white, beige, and brown adipose tissue biology, and highlights new mechanisms by which lactate participates in the maintenance of whole-body energy homeostasis. Lactate displays a wide range of biological effects in adipose cells not only through its binding to the membrane receptor but also through its transport and the subsequent effect on intracellular metabolism notably on redox balance. This study explores how lactate regulates adipocyte metabolism and plasticity by balancing intracellular redox state and by regulating specific signaling pathways. We also emphasized the contribution of adipose tissues to the regulation of systemic lactate metabolism, their roles in redox homeostasis, and related putative physiopathological repercussions associated with their decline in metabolic diseases and aging.

Keywords: lactate, redox metabolism, white adipocytes, beige adipocytes, brown adipocytes, adipose tissues, metabolic dialogs

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INTRODUCTION

Energy metabolism is based on redox (i.e., reduction and oxidation reactions) metabolism that corresponds to a finely tuned network of electron transfer between molecules. Oxidative catabolism releases electrons, which are accepted by coenzymes such as $NADP^+$ and NAD^+, which then become reduced (i.e., $NADPH,H^+$ and $NADH,H^+$; Wu et al., 2016a; Hosios and Vander Heiden, 2018; Xiao et al., 2018). Regenerating oxidized forms of coenzymes is critical to ensure proper metabolic activity (**Figure 1**). Electron assimilation, which corresponds to the storage of electrons within newly synthetized molecules (anabolism), and electron dissemination, which corresponds to the release of electrons within the environment, mainly through mitochondrial respiration and lactic acid fermentation, are keys for regenerating oxidized forms of coenzymes and maintaining redox homeostasis (**Figure 1**). In addition to supporting energy homeostasis, the redox state drives a variety of cellular functions, such as cell proliferation, differentiation, senescence, and secretory activity. The maintenance and adjustment of redox homeostasis are essential for normal cell and tissue function, and increasing evidence demonstrates that the redox state is the primary conductor regulating metabolic adaptation during stress (Gaude et al., 2018).

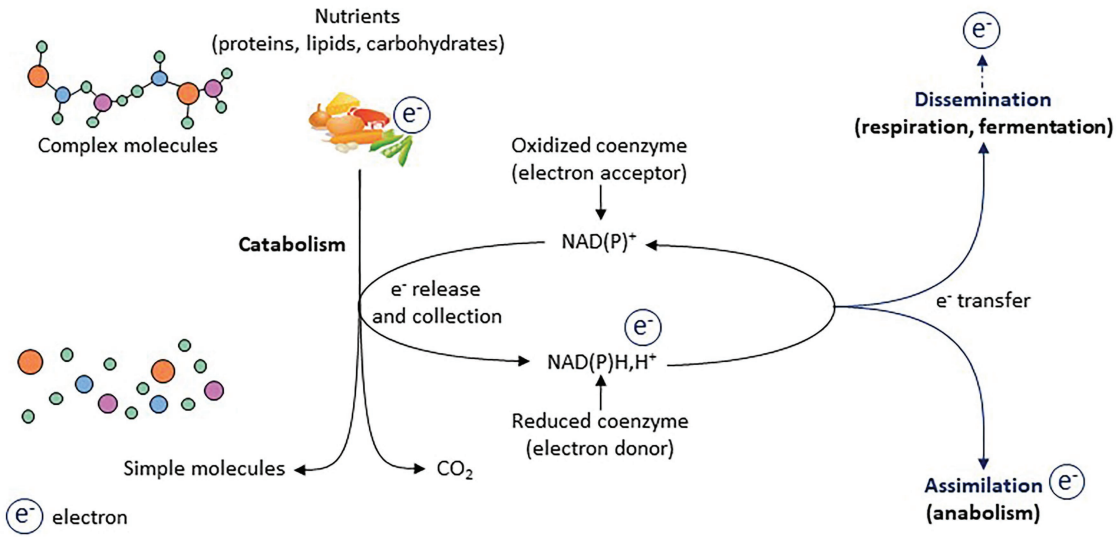

FIGURE 1 | Electrons as primary source of energy for cellular activity. Electrons represent the primary source of energy in cells. During catabolic processes, electrons are released from nutrients (which are oxidized) and are accepted by co-enzymes (which become reduced). Two processes are key for regenerating oxidized forms of co-enzymes and thus for redox homeostasis: the assimilation and dissemination processes.

However, redox metabolism is inevitably associated with electron leaks and reactive oxygen species (ROS) production, which, if not properly managed, can lead to oxidative stress and its related toxicities (Holmstrom and Finkel, 2014).

A massive cellular network of redox enzymes, couples, thiols, and metabolites supports electron flow between molecules that cooperate to maintain redox homeostasis. Among the different factors that can communicate metabolic and redox states between cells and organs, lactate plays a key role (Brooks, 2018; Ferguson et al., 2018; Rabinowitz and Enerback, 2020). In fact, the single and reversible reaction catalyzed by lactate dehydrogenase, which is submitted to the law of mass action and depends on the relative concentrations of lactate and pyruvate, directly regulates the cytosolic NAD^+/NADH ratio. As this redox reaction is close to equilibrium, the lactate/pyruvate ratio is considered as a marker of the cytosolic redox potential. Lactate production – which is mainly derived from glucose catabolism, although a small part can be obtained from alanine metabolism – and its export ensure redox homeostasis when the energy load overwhelms the oxidative capacities (**Figure 2**). Far from being a metabolic waste product, exported lactate can be consumed and oxidized either by neighboring cells or by organs at distance, making the link between glycolytic and oxidative pathways, and ensuring tissue and whole-body redox homeostasis (Brooks, 2018; Ferguson et al., 2018; Rabinowitz and Enerback, 2020).

Interorgan communication is key for maintaining whole-body energy and redox homeostasis on stresses and metabolic challenges (Castillo-Armengol et al., 2019). Among the different organs supporting such dialogs, the different types of adipose tissues (i.e., white, brown, and beige) play a key role (Cinti, 2012; Chouchani and Kajimura, 2019). In this study, we described how lactate can act as a redox signaling metabolite driving the fate of adipose cells and that lactate fluxes within adipose tissues may be key for whole-body redox homeostasis. Before exploring these aspects, we briefly described the main characteristics of lactate metabolism and adipose tissues.

LACTATE METABOLISM AS A KEY METABOLIC PATHWAY FOR THE MAINTENANCE OF REDOX HOMEOSTASIS

For many years, lactate has been viewed as a metabolic waste product, produced by glycolytic cells devoid of mitochondria or when glycolytic flux exceeds the oxidative capacities of cells. This historical view also associated lactate production with a potentially harmful effect, given the link between lactate and proton export, which can indeed contribute to microenvironment/blood acidification. However, lactate production is key for redox homeostasis and enables redox pressure disposal when the energy load overwhelms the oxidative capacities of a given cell (**Figure 2**), thereby limiting electron leaks and related oxidative stress. Once exported, lactate acts as a metabolic carrier ensuring the exchange of carbons and electrons between cells and organs. Intercellular and interorgan flows of lactate exist, and several collaborative lactate-dependent metabolic dialogs have been described as key for maintaining energy and redox homeostasis (Sonveaux et al., 2008; Pellerin and Magistretti, 2012; Rodriguez-Colman et al., 2017; Leveillard et al., 2019; Zhang et al., 2020).

From the Cell-Centric View…

In a glycolytic cell, lactate production from pyruvate and its export out of the cell are critical for intracellular redox homeostasis, in particular, when the glycolytic flux exceeds the

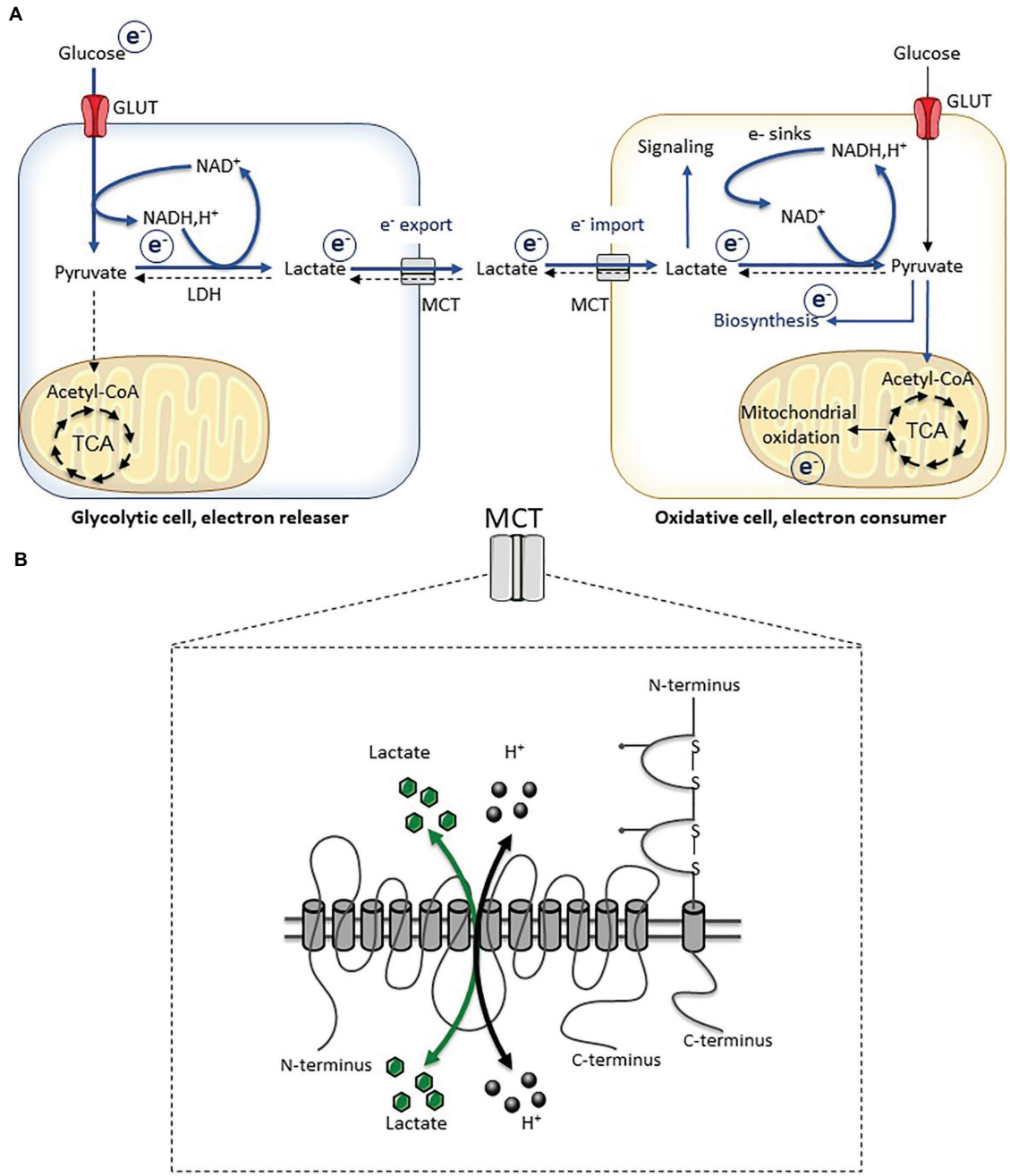

FIGURE 2 | Lactate transport and redox balance. **(A)** Inter-cellular lactate exchange is associated with inter-cellular electron fluxes. **(B)** Schematic representation of monocarboxylate transporters (MCT) which are constituted by 12 transmembrane domains, and their chaperone proteins (basigin and embigin). The transport of monocarboxylates (lactate, pyruvate, and ketone bodies) is associated with a proton transport (symport) and occurs bidirectionally depending on the electrochemical gradient. Adapted from Carriere et al. (2020). TCA, tricarboxylic acid cycle.

mitochondrial oxidative capacities. The reduction of pyruvate into lactate by the lactate dehydrogenase activity is associated with the oxidation of $NADH,H^+$ into NAD^+. This is critical for the activity of the glyceraldehyde dehydrogenase, which requires NAD^+ to catalyze a key step of the glycolytic pathway. Since the reaction catalyzed by lactate dehydrogenase activity is reversible and is submitted to the law of mass action, export of lactate is required to enable the reaction to further moving toward the lactate production. The transport of lactate is classically described as mediated by monocarboxylate transporters (MCT), especially the isoforms 1–4 that are considered as the main lactate (also pyruvate and ketone bodies) transporters (Halestrap, 2013; Perez-Escuredo et al., 2016). The transport of lactate is associated with the transport of proton and it is bidirectional

depending on the electrochemical gradient. The export of lactate through MCT, which massively occurs in a glycolytic cell, is therefore systematically associated with a simultaneous export of protons. Thus, the export of lactate does not solely correspond to an export of carbon and energy but rather to a crucial metabolic pathway for the maintenance of redox homeostasis in glycolytic cells (**Figure 2**). Besides the canonical MCT-dependent transport, alternative modes of lactate transport have recently been identified such as the SLC5A12 transporters in T lymphocytes (Pucino et al., 2019) or the connexin 43 in pancreatic cells (Dovmark et al., 2017).

…To the Ecosystem-Centric View

At the cellular level, lactate production is key to manage redox pressure. At the tissue or whole organism level, the cells able to consume lactate are essential to handle electron fluxes carried out by this metabolite and to maintain redox homeostasis. In fact, if lactate can appear as a metabolic waste for some cells, it is also a substrate for many other cells throughout the body. The quantification of metabolic fluxes using *in vivo* ^{13}C metabolite infusion experiments identified lactate as the main source of carbons fueling the Krebs cycle, in both physiological and pathological conditions (Faubert et al., 2017; Hui et al., 2017, 2020). Lactate also feeds biosynthesis pathways such as gluconeogenesis (Cori and Cori, 1929; Cori, 1981) and lipogenesis (Katz and Wals, 1974; Chen et al., 2016). The importance of lactate as a nutrient precursor has been demonstrated for almost 100 years when Carl Ferdinand and Gerty Theresa Cori demonstrated the role of muscle-produced lactate in hepatic gluconeogenesis (Cori and Cori, 1929; Cori, 1981). Whatever the metabolic pathways, when lactate is consumed by cells, this is associated with the import of electrons (**Figure 2**). In fact, lactate is oxidized into pyruvate by the lactate dehydrogenase activity and this is associated with the reduction of NAD^+ into $NADH,H^+$. Lactate-consuming cells must be able to manage these electron fluxes, through electron assimilation or dissemination processes. Mitochondrial activity corresponds to an electron dissemination process (**Figure 1**). If the electron load is in adequacy with the oxidative capacity of mitochondria, the production of mitochondrial ROS is low. However, if electron load overwhelms the oxidative capacities of mitochondria, the escape of electrons from the respiratory chain and the production of ROS are increased. Therefore, the rate of the lactate-dependent production of mitochondrial ROS results from the unbalance between the yield of electrons provided to the respiratory chain and the oxidative capacities, these latter depending on the quantity of mitochondria, the state of coupling/uncoupling, and the bioenergetics needs of the cells. Very interestingly, lactate by itself can activate specific signaling pathways to facilitate these electron management processes such as triggering mitochondrial biogenesis in muscle cells (Hashimoto et al., 2007) and inducing the expression of the uncoupling protein 1 (UCP1) in adipocytes (see the "Reciprocal relationships between lactate metabolism and adipose tissue biology" section; Carriere et al., 2014). These two cellular responses converge toward an increased oxidative potential, which will promote $NADH,H^+$ oxidation and NAD^+ regeneration.

THE MAIN ROLES OF ADIPOSE TISSUES IN WHOLE-BODY ENERGY AND REDOX HOMEOSTASIS

Among the different organs that cooperate to maintain whole-body energy homeostasis (Castillo-Armengol et al., 2019), adipose tissues are key not only because of their capacity to store the excess of energy under the form of triglycerides in white adipose tissues but also through the capacity of brown and beige adipose tissues to dissipate energy as heat (Chouchani and Kajimura, 2019). Lactate and redox metabolism play a critical role in the properties of each of these adipose depots (Carriere et al., 2020). Before highlighting the underlying mechanisms (see the "Reciprocal relationships between lactate metabolism and adipose tissue biology" section), following is a short description of their main characteristics.

White Adipocytes, From Energy Storage to Energy Release

White adipocytes are specialized cells with a unique ability to store energy into triglycerides during caloric intake and to release it in the form of fatty acids during periods of caloric deficit. Following chronic caloric excess, the expansion of white adipose tissue through the increase of adipocytes size (hypertrophy) and the formation of new adipocytes from progenitors (hyperplasia) contribute to the storage of energy in excess. At the opposite, when energy has to be made available for cellular activity, white adipocytes can mobilize their lipid stores through lipolysis to release energy (Lafontan and Langin, 2009).

Brown Adipocytes, Oxidation of Substrates, and Nonshivering Thermogenesis

Since its rediscovery in human adults in 2009 (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009), the contribution of brown adipose tissue to energy expenditure and metabolic health has been intensely studied (Sidossis and Kajimura, 2015; Betz and Enerback, 2018; Hussain et al., 2020). Brown adipocytes display significant oxidative capacities due to a large number of mitochondria and the presence of the mitochondrial UCP1 located in the mitochondrial inner membrane, which uncouples the oxidation processes from ATP synthesis (Cannon and Nedergaard, 2004). Cold exposure increases the expression of UCP1 and activates its uncoupling activity, in particular, by the fatty acids released during the sympathetic activation of lipolysis (Bertholet and Kirichok, 2017). Then, energy is dissipated as heat, at the expense of ATP synthesis (Nicholls and Locke, 1984).

Beige Adipocytes, Inducible on Stress in White Adipose Depots

Beige adipocytes display metabolic characteristics very similar to those of brown adipocytes, including UCP1 expression. However, they appear in specific regions of some white adipose tissues (Barreau et al., 2016; Dichamp et al., 2019) on metabolic

challenges such as cold exposure (Young et al., 1984; Loncar, 1991; Cousin et al., 1992) through distinct cellular mechanisms (Barbatelli et al., 2010; Lee et al., 2012; Wang et al., 2013; Park et al., 2021), and the brown-like phenotype disappears once the stress is over (Rosenwald et al., 2013). Notably, several UCP1-independent thermogenic mechanisms within beige (and brown) adipocytes have been recently uncovered (Roesler and Kazak, 2020). Although cold exposure is an important driver for beiging of adipose tissue, several other stimuli have been recently highlighted such as physical exercise (Bostrom et al., 2012; Dewal and Stanford, 2019), cancer-associated cachexia (Kir et al., 2014; Petruzzelli et al., 2014; Han et al., 2018), massive burn (Porter et al., 2015; Sidossis et al., 2015), and intermittent fasting (Li et al., 2017), but the role of beige adipocytes in these contexts remained to be clarified.

Adipose Tissues and Energy Homeostasis: A Redox View

One of the main characteristics of adipose tissues is their great plasticity, i.e., their ability to adapt their phenotype, metabolic activity, and function according to the nutritional status of the host and in response to metabolic challenges to ensure energy homeostasis (Chouchani and Kajimura, 2019). Besides this canonical view, one can consider adipose tissues as key regulators of electron fluxes, given their capacity to store electrons within triglycerides and to disseminate them, in white and brown/beige adipose tissues, respectively. In fact, in addition to carbon storage, the capacity of white adipocytes to store triglycerides can be viewed as a very efficient process of electron assimilation and storage, consistently with the reduced state observed during adipogenesis (Galinier et al., 2006). Since the high oxidation of $NADH,H^+$ into NAD^+ occurs in the presence of activated UCP1, brown and beige adipocytes may also play a key role in the redox balance by acting as controllable electron dissemination systems. These intimate relationships between adipose tissues and redox metabolism are strengthened by the growing evidence that lactate metabolism can fine-tune adipose tissue biology according to the metabolic conditions and that, reciprocally, adipose tissues impact systemic lactate metabolism.

RECIPROCAL RELATIONSHIPS BETWEEN LACTATE METABOLISM AND ADIPOSE TISSUE BIOLOGY

In addition to the well-known impact of adipose tissues on glucose and lipid systemic homeostasis, recent findings highlight them as regulators of other circulating metabolites, such as succinate (Mills et al., 2018, 2021) or branched-chain amino acids (Yoneshiro et al., 2019). Several metabolites have also been shown to promote adipocyte plasticity toward a beige phenotype (Sahuri-Arisoylu et al., 2016; Liu et al., 2020). In the following section, we focused on the relationships between lactate metabolism and metabolic activity of adipose tissues, highlighting that lactate can act as a signaling molecule in adipose tissues and that, reciprocally, adipose tissues can impact systemic lactate metabolism.

Lactate Regulates the Metabolic Activity of White, Brown, and Beige Adipocytes

White adipocytes convert a significant part of the metabolized glucose into lactate (Hagstrom et al., 1990; DiGirolamo et al., 1992; Krycer et al., 2019). The magnitude of the glucose conversion to lactate can reach 50–70% of total glucose metabolized (DiGirolamo et al., 1992). Several signaling pathways regulate this glycolytic pathway such as the insulin-dependent signaling, the FOXK1/2 transcription factors being recently highlighted as important molecular mediators (Sukonina et al., 2019). In addition to adipocytes, the stromal vascular fraction of white adipose tissues is also an important lactate producer, as demonstrated in cells isolated from several rat adipose tissues (Rotondo et al., 2019). Lactate regulates white adipose cell biology and metabolic activity through paracrine and autocrine effects, by acting as a metabolic substrate feeding lipogenesis as a lipid precursor (Katz and Wals, 1974; Francendese and Digirolamo, 1981; Chen et al., 2016) and by stimulating the adipogenic differentiation program of preadipocytes, as recently demonstrated (Harada et al., 2018). This is consistent with the increase in the MCT expression along white adipocyte differentiation (Petersen et al., 2017). Lactate also inhibits the lipolytic activity of white adipocytes by binding to the Gi-coupled G protein-coupled receptor (GPR81; Ahmed et al., 2010). In brief, lactate effects are linked to those of insulin, one of the main lipolysis inhibitors. Insulin increases glucose utilization, a large part of this glucose is converted into lactate that is exported. In an autocrine/paracrine manner, lactate binds to GPR81 that – through its effects on adenylate cyclase – induces a decrease in the level of cAMP and therefore lipolysis (Ahmed et al., 2010; **Figure 3**). Lactate might thus be observed as a metabolic signal, promoting white adipogenesis and anabolism, to increase the energy storage capacities of white adipose tissues.

Despite a very different metabolic profile and much greater oxidative capacities, brown adipose tissue is also a major site of lactate production. Although glucose consumption feeds the oxidative metabolism of brown adipose tissue (Wang et al., 2020b), a large amount of this glucose is converted into lactate and exported (Ma and Foster, 1986; Schweizer et al., 2018; Weir et al., 2018), in particular during cold/noradrenergic stimulation. Intense glycolysis ensured by the export of lactate may feed multiple metabolic pathways in brown adipocytes activated by cold, including ATP production (i.e., to compensate for the very low mitochondrial ATP production), the pentose phosphate pathway (i.e., redox balance and lipid synthesis), and glycerol production (i.e., lipogenesis; Hankir and Klingenspor, 2018). Stimulation by the β3 adrenergic pathway also increases lactate release by beige adipocytes in an MCT1-dependent manner, and this lactate release is required for the efficient utilization of glucose by beige adipocytes (Lagarde et al., 2020; **Figure 4**). This is due to the key role of lactate production and export for redox homeostasis, as discussed earlier. The importance of redox homeostasis for thermogenic adipocytes has been recently highlighted (Nguyen et al., 2020).

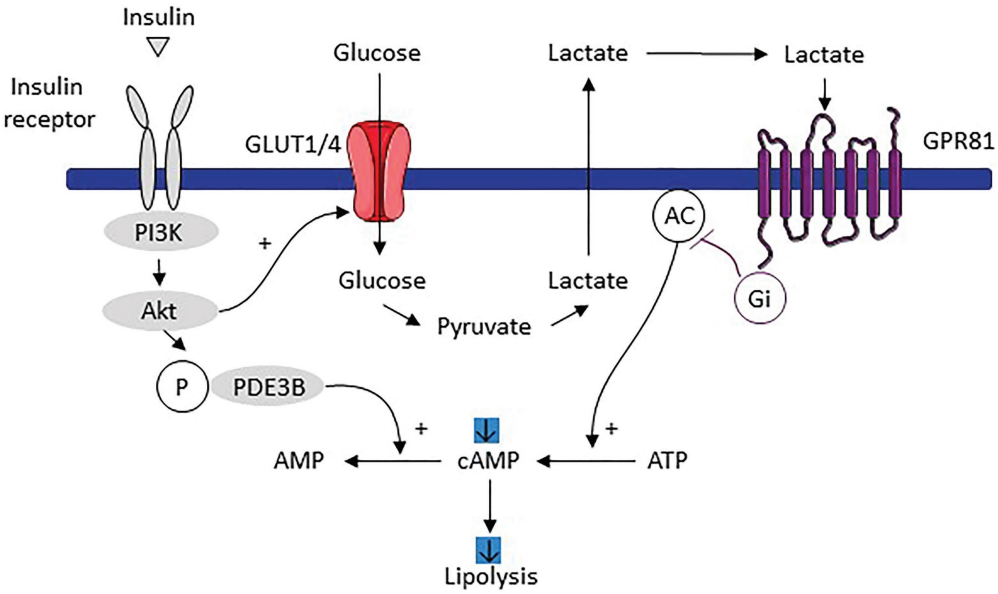

FIGURE 3 | Mechanism of lipolysis inhibition induced by adipocyte dependent lactate production in response to increased glucose utilization stimulated by insulin. Imported glucose following insulin signaling is converted to lactate and then exported. By an autocrine/paracrine action, lactate activates the GPR81 receptor which, through its effects on adenylate cyclase, decreases the level of cAMP and therefore lipolysis. Adapted from Ahmed et al. (2010). PI3K, phosphoinositide 3-kinase; POE3B, phosphodiesterase 3B; AMPc, cyclic adenosine monophosphate; ATP, adenosine triphosphate; AC, adenylate cyclase; and GPR81, G protein-coupled receptors associated with inhibitory regulative G-protein (Gi).

Further highlighting the importance of MCT1-dependent lactate fluxes in beige adipocyte biology, this transporter is expressed by the subpopulation of adipocytes that turn UCP1 positive on cold exposure, making of it as a marker of inducible beige adipocytes (Lagarde et al., 2020). This finding is very reminiscent of what is occurring during the postnatal development of brown adipose tissue, since only adipocytes expressing MCT1 give rise to UCP1$^+$ mature brown adipocytes in Syrian hamster (Okamatsu-Ogura et al., 2018). MCT1 is also strongly expressed in brown adipocytes of adult mice (Iwanaga et al., 2009) and supports optogenetically induced nonshivering thermogenesis (Jeong et al., 2018) through the transport of lactate into mitochondria (Jeong et al., 2018).

Concomitantly to the net lactate outgoing flux and even when glucose is not limiting, a significant import of lactate occurs through MCT1, as shown by ^{13}C lactate isotopic tracing experiments. From the thermodynamic point of view, lactate exchange through the plasma membrane is representing the transmembrane equilibration of the respective electrochemical gradients of the transported species (i.e., lactate and protons in this case) in the two compartments through the lactate transporter(s). In other words, the transport is reversible, which means that it does operate in the two directions at the same time with a net flux in one particular direction. Importantly, the imported lactate feeds the oxidative metabolism of beige adipocytes (Lagarde et al., 2020), as also shown for white and brown adipose tissues (Hui et al., 2017, 2020). However, the noradrenergic pathway does not increase lactate uptake in beige adipocytes but fosters lactate release, as a result of high increase in glycolytic flux (Lagarde et al., 2020). Glucose might be then preferred over lactate as a circulating substrate feeding thermogenesis during cold exposure. In response to an increase in systemic lactatemia, the import of lactate may be favored in beige adipocytes. The physiological situations where lactate utilization is increased in beige adipocytes to promote their oxidative metabolism are unknown to our knowledge. In addition to the regulation of the metabolic activity of adipocytes and independently of the β3 adrenergic signaling, intracellular lactate regulates the expression of UCP1 by acting on the intracellular redox balance, thereby contributing to the beiging of adipose tissues.

Lactate as a Signaling Molecule Inducing Beiging

In addition to its role as an energy substrate supporting the oxidative metabolism of beige and brown adipocytes, lactate acts as a signaling molecule triggering their development (Carriere et al., 2014; **Figure 5**). This effect is independent on the lactate receptor GPR81 but requires redox modifications due to the transport of lactate by MCT1 and increased NADH,H$^+$/NAD$^+$ ratio subsequent to the lactate conversion into pyruvate by lactate dehydrogenase. Notably, lactate–UCP1 signaling does not occur in the presence of an uncoupling agent (i.e., a state equivalent to activated UCP1), very consistently with the role of NADH,H$^+$/NAD$^+$ ratio as a metabolic sensor regulating UCP1 expression. Since UCP1-dependent uncoupling accelerates the activity of the respiratory chain by therefore facilitating NADH,H$^+$ oxidation and the overall electron flow, we proposed that UCP1-dependent uncoupling may be key for redox stress management (Carriere et al., 2014; Jeanson et al., 2015; **Figure 5**).

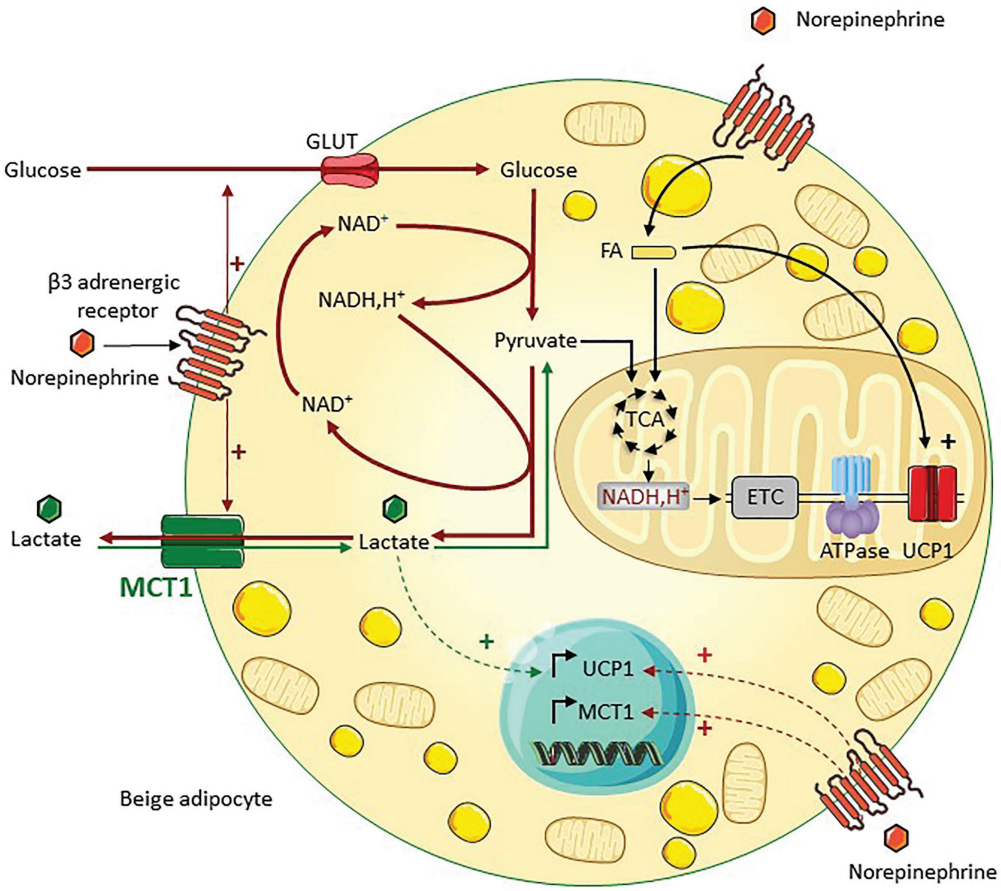

FIGURE 4 | MCT1 is a key regulator of lactate bidirectional fluxes in beige adipocytes. MCT1, which is expressed at the cell surface of beige adipocytes, sustains bidirectional lactate fluxes. Lactate export, which is predominant under noradrenergic stimulation such as cold exposure, supports glycolysis through NAD^+ regeneration (red arrows). Concomitant lactate import occurs (green arrows), which fuels the oxidative metabolism and promotes the induction of UCPl, thereby increasing the oxidative capacity of beige adipocytes (adapted from Lagarde et al., 2020). ETC, electron transport chain; ATPase, ATP synthase; and FA, fatty acids.

This is supported by findings demonstrating that the uncoupling activity of UCP1 is activated by mitochondrial ROS (Chouchani et al., 2016) and that UCP1 controls ROS in brown adipose tissue (Jastroch, 2017). Lactate-induced UCP1 expression could represent an adaptive mechanism where adipocytes acquire increased oxidative capacities to maintain redox homeostasis. The role of lactate as a beiging inducer has been shown in different contexts (**Figure 6**). The implication of intracellular lactate production in UCP1 induction has been demonstrated in preadipocytes (Bai et al., 2016) and in muscle cells (Kim et al., 2017). It has also been demonstrated that during intermittent fasting, the reprogramming of the intestinal microbiota induces lactate production that contributes to the beiging of white adipose tissue, which plays an important role in the improvement of metabolic profiles (Li et al., 2017). The alteration of lactate-UCP1 signaling has physiopathological consequences, as the inhibition of lactate production through interferon regulatory factor-3 (IRF3)-mediated inhibition of lactate dehydrogenase contributes to the downregulation of UCP1 expression and thermogenesis, in an inflammatory context (Yan et al., 2021). Recently, in a mouse model exhibiting a mutation in the skeletal muscle Ca^{2+} release channel (i.e., RYR1) that induces malignant hyperthermia, an increased production of lactate in muscle is involved in the beiging of white adipose tissue and the activation of brown adipose tissue in an MCT1-dependent manner, which would actively contribute to the hyperthermic phenotype (Wang et al., 2020a). Together, these recent studies highlight the physiological and pathological importance of lactate-induced beiging. In addition, to promote beiging, lactate also induces the expression and secretion of the fibroblast growth factor-21 in both adipocytes (Jeanson et al., 2016) and muscle cells (Villarroya et al., 2018), which is an important regulator of glucose homeostasis and which triggers adaptive responses to reduce metabolic stresses. Thus, lactate, by various independent mechanisms, increases the oxidative capacities of beige adipocytes and stimulates the release of factors enabling adaptation to redox pressure and metabolic stresses.

All these findings highlight how lactate metabolism plays a role in the biology, metabolism, and plasticity of adipose tissues, by acting as a redox stress signal enabling cell adaptation. Interestingly, several studies of the literature show that adipose

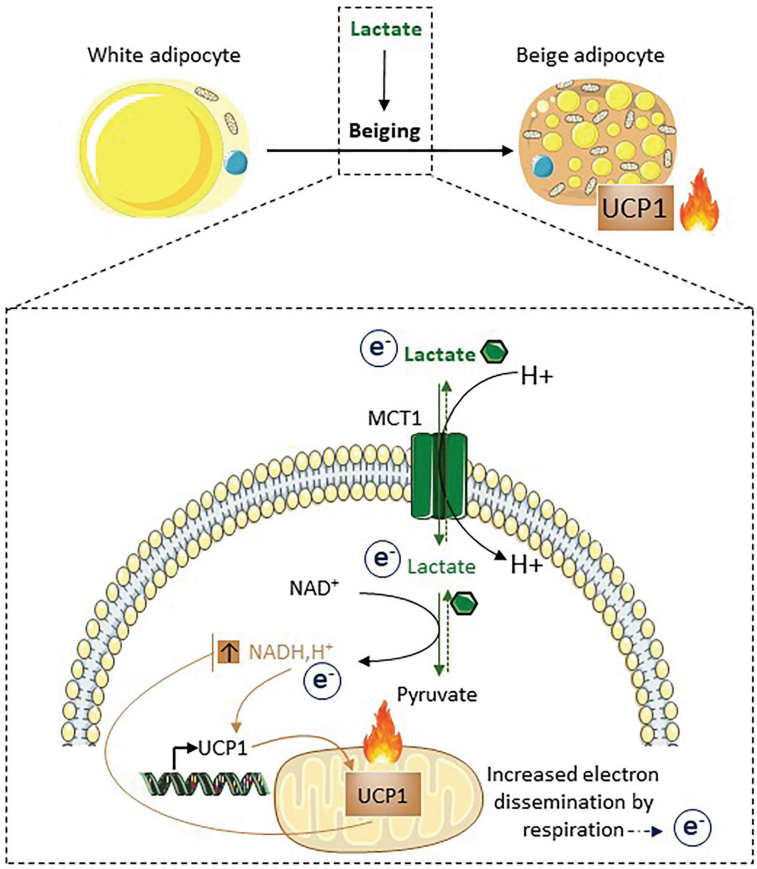

FIGURE 5 | Lactate induces beiging as a way to dissipate redox pressure. Following its import through MCT1, lactate is converted into pyruvate. This conversion is associated with the reduction of NAD^+ into $NADH,H^+$. The increased NADH, H^+/NAD^+ ratio triggers UCP1 expression. Due to the properties of UCP1 and its effects on the respiratory chain, UCP1-dependent uncoupling accelerates oxidation of $NADH,H^+$ into NAD^+. Thus, UCP1-dependent uncoupling, in addition to its involvement in non-shivering thermogenesis, may also play an active role in redox homeostasis. Of note, MCT1 is expressed at the cell surface of the subpopulation of adipocytes that will express UCP1 after cold exposure, highlighting it as a marker of inducible beige adipocytes (Carriere et al., 2014; Jeanson et al., 2015; Lagarde et al., 2020).

tissues can regulate systemic lactate metabolism, opening physiopathological perspectives.

Contribution of Adipose Tissues to the Regulation of Systemic Lactate Metabolism

Several tissues contribute to the regulation of lactate systemic levels, such as muscles, liver, red blood cells, brain, and heart, the rate of lactate release and uptake depending on the physiopathological situations for each tissue (Adeva-Andany et al., 2014). It is known that white adipose tissues are an important site of lactate production that can impact lactate circulating levels (DiGirolamo et al., 1992), probably due, at least in part, to the fact that they represent an important percentage of the total body weight. The quantitative contribution of all adipose depots to total systemic lactate metabolism is, however, unclear and not resolved to our knowledge. Due to the insulin-dependent stimulation of glucose uptake, lactate release by white adipose tissue increases in a postabsorptive state and is likely a major contributor to the increased circulating levels of lactate following insulin or glucose challenge in healthy humans (Hagstrom et al., 1990; Jansson et al., 1994; Qvisth et al., 2007). Lactate release by adipose tissues is further enhanced in elderly and obese subjects (Jansson et al., 1994; Faintrenie and Geloen, 1997) due to their high-fat mass and the decrease in their oxidative capacities, notably due to hypoxia (Trayhurn and Alomar, 2015). Fasting plasma lactate concentration is increased in obese subjects compared with lean control patients, and also in diabetic patients (Lovejoy et al., 1992; Adeva-Andany et al., 2014), which might be an early change in the time course of the disease (Wu et al., 2016b). These results are in agreement with the positive correlation observed between the size of adipocytes and the quantity of lactate produced (DiGirolamo et al., 1992). In the context of obesity, hypertrophic adipocytes with extremely limited oxidative potential and saturated storage capacities are less able to use glucose as a carbon source for oxidative metabolism. Glucose is then massively converted into lactate and exported. Although the role of lactate in the development of insulin resistance needs to be further investigated, lactate

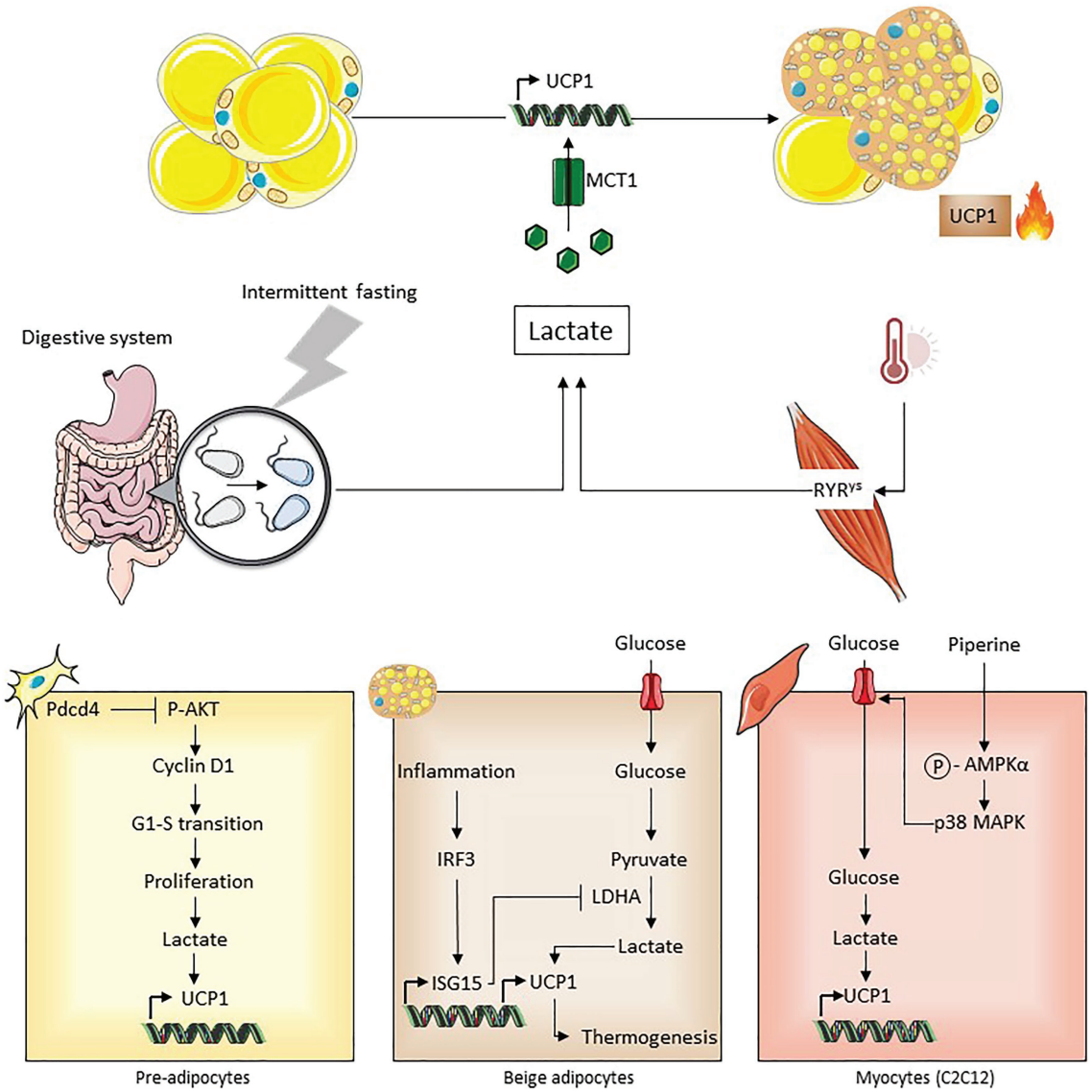

FIGURE 6 | Different contexts associated with lactate-induced beiging. *In vivo*, reprogramming of gut microbiota following intermittent fasting is associated with increased lactate production, which may contribute to beiging that improved systemic metabolic parameters (Li et al., 2017). Increased muscle lactate production caused by a calcium channel mutation is associated with beiging (Wang et al., 2020a). Intracellular production of lactate induced by pdcd4 and piperine in preadipocytes and C2C12 cells respectively, upregulates UCPl expression (Bai et al., 2016; Kim et al., 2017). IRF3/ISG15-mediated inhibition of lactate dehydrogenase decreased lactate-induced UCPl expression thus impairing thermogenesis during inflammation (Yan et al., 2021).

could compromise insulin signaling and glucose transport in muscle (Choi et al., 2002). These results suggest that in the context of obesity or aging, the alteration of the oxidative potential of white adipose tissues may increase lactate production by adipocytes, which might participate in the development of metabolic diseases. Using MCT1 heterozygous mice that are resistant to obesity (Lengacher et al., 2013), the transport of lactate into hepatocytes has been shown to contribute to the development of hepatic steatosis (Carneiro et al., 2017), suggesting that during obesity, dysregulated lactate metabolism may participate to the development of metabolic disorders. Among the underlying mechanisms, the acetylation of lactate dehydrogenase B in the liver of high-fat-diet-exposed mice would contribute to impaired lactate clearance and liver steatosis (Wang et al., 2021). Notably, the decrease of beiging in the obesity-associated lactate-rich environment could be due to several mechanisms such as the loss of cellular plasticity and/or the alteration of lactate-sensing mechanisms and impaired lactate fluxes in adipose cells in an obesity context. Although genetic models are definitively required to better investigate the role of adipose tissues in the regulation of lactate systemic lactate fluxes, a transgenic Drosophila model harboring the inhibition of lactate dehydrogenase specifically in the fat body has been recently generated. Very interestingly, these flies

exhibited a decrease in circulating levels of lactate, which was associated with an improved body-wide glucose utilization (Krycer et al., 2019), further highlighting the role of adipose tissues in whole-body energy homeostasis through their impact on the circulating levels of lactate.

The role of brown and beige adipose tissues on the regulation of systemic lactate metabolism is poorly described. However, physical exercise that is associated with increased lactate circulating levels induces beiging (Bostrom et al., 2012; Dewal and Stanford, 2019) and increases the expression of MCT1 in brown and beige adipose tissues (De Matteis et al., 2013). This could therefore increase the rate of lactate utilization in these tissues, as a way to manage redox pressure and related oxidative stress. Whether beige and brown adipocytes could act as lactate metabolic sinks as recently proposed for succinate (Mills et al., 2018, 2021) or branched-chain amino acids (Yoneshiro et al., 2019) remain to be demonstrated. This hypothesis may be supported by the positive correlation between plasma lactate and body temperature in five healthy volunteers subjected to physical exercise (Son'kin et al., 2014). Interestingly, the rise in blood lactate levels precedes the rise in temperature. In this study, the highest body and local temperatures at the nape of the neck (i.e., the location of brown adipose tissue in humans) as well as the highest blood lactate levels were observed not during exercise but within minutes afterward. We could envision that during exercise, some of the lactate produced is consumed by the muscle and heart, but that when exercise is stopped, the activation of brown adipose tissue could limit the rise in blood lactate levels.

Conclusion and Perspectives

Neglected during many years and even considered as a metabolic waste, the multiple roles of lactate as metabolic substrate, redox shuttle, and signaling molecule participate to its rehabilitation as a hub for multiple metabolic pathways notably in adipose tissues. At the whole-body level, the capacities to maintain redox homeostasis on challenges are key for energetic homeostasis. Given their important mass and their high plasticity, adipose tissues appear to be major players in the redox balance and are perfectly adapted to respond to acute and chronic redox stresses. Adipose tissues may act as a redox buffering system by assimilating or disseminating electrons depending on the situation and regulating systemic redox homeostasis through interorgan metabolic dialogs. Although most cells of the organism are capable of transporting lactate, the significant capacities of adipocytes to store energy in excess (i.e., white adipocytes) or to dissipate it in the form of heat (i.e., beige and brown adipocytes) and their high cellular and metabolic plasticity make them cells that are specially adapted to play this redox buffering function. Lactate-dependent cellular responses may be part of the stress-responsive mechanisms enabling adipose cells to acquire an appropriate phenotype to face stresses. Any lack of appropriate response to lactate challenge could have tissue and whole-body repercussions. It is known that in a context of obesity or aging, due to mitochondrial dysfunctions, a decrease in adrenergic tone and modification of inflammatory state, plasticity of adipose tissues, their storage capacity, and their oxidative capacities are reduced. In these pathological contexts, adipose tissues may no longer allow the body to face redox stress and could even contribute to it by increasing lactate release. Investigating the existence of new interorgan communication, which is mediated by lactate or additional metabolites involved in redox homeostasis, and their role during aging or metabolic diseases is definitively an exciting area for future studies.

AUTHOR CONTRIBUTIONS

DL designed all the figures. AC supervised and finalized the manuscript. DL, YJ, J-CP, AG, IA, LC, and AC contributed to the design and writing of the manuscript, proofread, and gave comments and suggestions. All authors contributed to the article and approved the submitted version.

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frontiers in Physiology

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The Role of cAMP-PKA Pathway in Lactate-Induced Intramuscular Triglyceride Accumulation and Mitochondria Content Increase in Mice

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The glycolytic product of exercise, lactate, has long been recognized to promote lipid accumulation by activation of G-protein-coupled receptor 81 (GPR81) and inhibition of the cyclic adenosine monophosphate-protein kinase A (cAMP –PKA) pathway in adipose tissue. Whether lactate causes a similar process in skeletal muscle is unclear. Lactate might also improve mitochondria content in skeletal muscle; however, the mechanism is not clarified either. In this study, using intramuscular injection of lactate to the gastrocnemius and intraperitoneal injection of forskolin (activator of cAMP-PKA pathway), we identified the role of the cAMP-PKA pathway in lactate-induced intramuscular triglyceride accumulation and mitochondrial content increase. The intramuscular triglyceride level in the gastrocnemius increased after 5 weeks of lactate injection ($p < 0.05$), and this effect was blocked by forskolin injection ($p < 0.05$). Corresponding expression level changes of GPR81, P-PKA/PKA, P-CREB/cAMP-response element binding protein (CREB), and proteins related to lipid metabolism suggest that lactate could induce intramuscular triglyceride accumulation partly through the inhibition of the cAMP-PKA pathway. Meanwhile, the intramuscular expression of citrate synthase (CS) and the activity of CS increased after 5 weeks of lactate injection ($p < 0.05$), but the change of CS expression was not blocked by forskolin injection, suggesting other mechanisms might exist. Consequently, exploration for other potential mechanisms that might contribute to the lactate-induced mitochondria content increase was conducted. We found an increase in the contents of lactate-related metabolites in skeletal muscle mitochondria after acute lactate injection (the *p-value* of each analysis is less than 0.05). LHDA was also validated to exist in mitochondria in this study. These results provide a possibility for metabolism-related mechanisms of lactate-induced mitochondria content increase. Future study is needed to validate this hypothesis. In conclusion, lactate-induced intramuscular triglyceride accumulation is achieved by inhibition of lipolysis, and this process

is regulated by the cAMP-PKA pathway. Promoted lipogenesis also contributes to lactate-induced triglyceride accumulation, and this process might also be regulated by the cAMP-PKA pathway. Lactate injection might increase mitochondria content and cAMP-PKA pathway might have a limited contribution, while other metabolism-related mechanisms might play a prominent role.

Keywords: lactate, cAMP, intramuscular triglyceride, mitochondria content, skeletal muscle

INTRODUCTION

Lactate has long been recognized to promote lipid accumulation in adipocytes. Infusion of lactate has been reported to suppress lipolysis in adipose tissue (Gold et al., 1963; Bjorntorp, 1965; Houghton et al., 1971; Boyd et al., 1974; De Pergola et al., 1989; Cai et al., 2008). This effect of lactate is known to be correlated with the activation of its receptor G-protein-coupled receptor 81 (GPR81) and the inhibition of its downstream cAMP-PKA pathway in adipose tissue (Sakurai et al., 2014). Our recent research showed that lactate could directly induce similar lipid accumulation in skeletal muscle as it does in adipose tissue (Zhou et al., 2021b). However, the mechanism is unclear.

Previous studies have validated that intramuscular triglyceride accumulation is usually accompanied by mitochondrial adaption to breakdown triglycerides more efficiently, and a positive association has also been found between intramuscular triglyceride accumulation and the expression levels of mitochondrial biogenesis proteins (Koves et al., 2013). Growing evidence suggests that lactate might act as a signaling molecule and contribute to mitochondrial adaptation in skeletal muscle (Takahashi et al., 2020). Our recent research also verified that chronic lactate injection could not only induce the intramuscular triglyceride accumulation but also increase the expression levels of mitochondria protein, suggesting increased mitochondria content after lactate administration (Zhou et al., 2021b). However, the mechanism of this lactate-induced mitochondria content increase in skeletal muscle is rarely investigated.

It is reported that mitochondria content might be modulated by the cAMP-PKA pathway. An experiment *in vitro* indicated that activation of the cAMP-PKA pathway increased the copy number of mitochondrial DNA (Bogacka et al., 2005). De Rasmo et al. (2012, 2015) also demonstrated that the cAMP-PKA pathway could regulate the expression levels of the subunits of the electron transport chain complex in mitochondria. However, in our previous study, lactate injection suppressed cAMP-PKA pathway and upregulated the expression level of PGC1-α and CS, which seems to be paradoxical. In this study, the expression level of CS and its activation were also upregulated by lactate, and increased expression of CS was not inhibited by forskolin. Hence, we presume that mechanisms other than cAMP-PKA pathway might play a prominent role in lactate-induced mitochondria content increase.

As indicated above, the mechanism whereby lactate regulates intramuscular lipid accumulation and mitochondria content is presently unclear. Here, using intramuscular injection of lactate to the gastrocnemius and intraperitoneal injection of forskolin (activator of cAMP-PKA pathway), we investigated the role of the cAMP-PKA pathway in lactate-induced intramuscular lipid accumulation and mitochondria content increase. Other possible mechanisms of increased mitochondria content after lactate injection were also explored from the aspect of energy metabolism.

MATERIALS AND METHODS

Animals

Experiments were performed with c57bl/6 mice (7 weeks of age, obtained from Chengdu DaShuo Biological Technology Co., Ltd., China). Mice were maintained on a standard rodent chow diet and water *ad libitum* under 12-h light and dark cycles. All the administration was implemented after an acclimation period of 1 week.

In the first experiment aiming to clarify the time dependence of acute lactate and forskolin injection-induced variation of cAMP-PKA pathway, c57BL/6 mice were randomly assigned into 10 groups: sacrificed after acute phosphate-buffered saline injection (AP; $n=6$); sacrificed after acute DMSO injection (AD; $n=6$); sacrificed at 15, 30, 60, and 120 min after acute lactate injection (AL-15, AL-30, AL-60, and AL-120; $n=6$); sacrificed at 15, 30, 60, and 120 min after acute forskolin injection (AF-15, AF-30, AF-60, and AF-120; $n=6$); sacrificed at 60 min after acute lactate and forskolin injection (60 min interval, ALF; $n=6$).

In the subsequent chronic experiment, the animals were assigned randomly into five groups: chronic phosphate-buffered saline treated group (CP; $n=8$); chronic lactate treated group (CL; $n=8$); chronic forskolin treated group (*CF*; $n=8$); chronic DMSO treated group (CD; $n=8$); and chronic lactate and forskolin treated group (CLF; $n=8$). Each chronic group was administrated for 5 weeks and sacrificed by cervical dislocation at 72 h after the last injection. No death occurred during the experiment.

The procedures for the care and use of animals were approved by the Ethics Committee of Sichuan University and conform with all the applicable institutional and governmental regulations on the ethical use of animals.

Treatment Protocol

Lactate treatment was performed with sodium lactate (C43922, Acros) solution prepared in PBS (C0221A, Beyotime). Local delivery of sodium lactate solution was performed by intramuscular injection in the gastrocnemius of the left limb (0.64 ml/kg). According to a previous study

(Nikooie and Samaneh, 2016), this value of lactate (0.25 M) was selected to elevate lactate levels in the gastrocnemius muscle to an average value of 25 mmol/L. PBS treatment was performed with PBS solution at the volume of the lactate solution and by the same injection method of the lactate treatment. Forskolin treatment was performed with 5% forskolin (F3917, SigmaAldrich) solution prepared in 4% DMSO. The delivery of forskolin solution was performed by intraperitoneal injection (5 mg/kg). DMSO treatment was performed with 4% DMSO solution at the volume of the forskolin solution and by the same injection method of the forskolin treatment. Lactate and forskolin treatment was performed with lactate solution and then forskolin solution following the same injection protocols mentioned above. The time interval between the two injections is 15 min.

Injections in chronic treatment groups were implemented five times a week (one injection daily for consecutive 2 days, after that comes a day off; and then one injection daily for consecutive 3 days, after that comes a day off) for 5 weeks, and the injection in the first experiment was carried out with the same procedure of every single injection in the chronic groups.

Determination of Blood Lactate Concentration

Portable electrochemical devices Lactate Scout and Sensors (SensLab EKF, Germany) were used to determine the blood lactate level after acute lactate injection. A previous study has proved the accuracy of this device (Romanov et al., 2020). Blood drop was excised from the aseptically treated wound of mice's tail. After resting for 20 min, resting blood lactate concentration was determined. Then mice were injected with lactate and then placed in the chamber to avoid external stimuli for the subsequent tests.

Oil Red O Staining of Gastrocnemius

To determine the morphological effects of lactate on intramuscular lipid droplets, oil red O staining was performed using frozen tissue sections as previously described. Briefly, muscle tissue was washed with PBS, treated with 4% paraformaldehyde, and incubated in 60% oil Red O for 10 min. After washing residual oil red O from muscle tissue, images were collected using an inverted microscope (NIKON Eclipse Ci).

Immunofluorescence of Gastrocnemius

According to a previous study (Elustondo et al., 2013), the muscle of mice was fixed in 4% paraformaldehyde for 1 h, treated with 100 mM glycine for 30 min, and permeabilized with Tris-buffered saline containing 0.1% Tween 20 (w/v) for 20 min. The samples were blocked for 1 h with PBS containing 1% gelatin, incubated with anti-LDHA (Proteintech, 19987-7-AP) for 1 h at room temperature, washed four times with PBS, and then incubated with the secondary antibody goat anti-rabbit conjugated with Dylight 549 for another hour (711-505-152, Jackson ImmunoResearch Laboratories). Following washes, the same samples were incubated with anti-HSP60 (Invitrogen, MA3-012) for 1 h at room temperature, washed four times with PBS, and then incubated with the secondary antibody goat anti-rabbit conjugated with Dylight 549 for another hour, and then washed and mounted with DAKO mounting media. Images were collected using a fluorescent confocal microscope (NIKON Eclipse Ti). Experiments were repeated three times in triplicate.

Triglyceride Assay of Gastrocnemius

Intracellular triglycerides were assayed using a triglyceride assay kit (GPO-POD; Applygen Technologies Inc., Beijing, China). About 25 ± 5 mg of the gastrocnemius muscle of six mice of each group were weighed and lysed on ice. After 70°C heating for 10 min, each sample was placed in a 96-well plate with two duplicates and then mixed with the kit's A + B solution. After a 15 min incubation at 37°C and cooling to room temperature, the resultant purple color is measured using a spectrometer at 492 nm. Then the final values are normalized by each sample's protein concentration measured by the BCA assay.

Citrate Synthase Activity Assay of Gastrocnemius

Citrate synthase (CS) activity was assayed using a citrate synthase kit (Beijing Solarbio Science & Technology Co., Ltd., China). About 25 ± 5 mg of the gastrocnemius muscle of eight mice of each group were weighed and lysed on ice and then analyzed according to the protocol provided by the kit. The final values are normalized by each sample's protein concentration measured by the BCA assay.

Lactate Concentration Assay of Gastrocnemius

Lactate concentration of gastrocnemius after acute lactate injection was assayed using a lactate acid assay kit (Beijing Solarbio Science & Technology Co., Ltd., China). About 35 ± 5 mg of the gastrocnemius muscle of four mice of each group were weighed and lysed on ice and then analyzed according to the protocol provided by the kit. The final values are normalized to each sample's fresh weight.

Liquid Chromatography-Mass Spectrometry of Gastrocnemius

At 5 min after acute injection of lactate and PBS, mice were sacrificed and mitochondria were obtained using the Mitochondria Isolation Kit (Cat: KGA827, KeyGEN, Nanjing, China): (1) Left gastrocnemius muscle tissue was collected and washed with PBS. The tissue was minced and placed in a glass homogenizer, to which was added the pre-cooled Lysis Buffer (6-fold volumes of the tissue), and then dounced 20 times with the glass homogenizer in an ice bath; (2) Homogenate was transferred into microcentrifuge tubes containing 0.2 ml Medium Buffer, mixed gently and then centrifuged for 5 min at 1,200 × *g* and 4°C and the supernatant was collected. The supernatant of the first centrifuge was centrifuged once more and the supernatant was collected; and (3) The supernatant was transferred to a clean microcentrifuge tube. The supernatant was centrifuged at 7,000 × *g* for 10 min, and the supernatant was discarded. The precipitates were mitochondria.

Mixed with MeOH-H2O (8:2), mitochondria was stored at −20°C for 20 min and ultrasonicated in an ice bath for 15 min. The viscous mixture was centrifuged at 13,300 rpm for 15 min at 4°C. Then the centrifuged supernatant was collected and concentrated under high vacuum for 2 h. After stored at −80°C for 48 h, the concentration was redissolved in MeOH-H2O (8:2) with 13C1-Lactate. The solution was ultrasonicated in an ice bath for 10 min and centrifuged at 13,300 rpm for 10 min at 4°C. The centrifuged supernatant was collected for mass spectrometry (ms).

Contents of L-lactic acid, succinic acid, L-malic acid, and oxalacetic acid were analyzed on an AB SCIEX QTRAP 5500 triple quadrupole mass spectrometer (AB SCIEX, Framingham, United States) equipped with a Shimadzu LC-30AD HPLC system (Shimadzu, Kyoto, Japan). About 1 μl of the mitochondria extract was injected onto a Waters Acquity UPLC HSS-T3 columna held at 30°C for chromatographic separation. The mobile phase A was water with 0.1% formic acid and mobile phase B was methanol, and gradient elution was performed. The flow rate was 0.3 ml/min. All the samples were kept at 4°C during analysis.

The mass spectrometer was operated in negative ion mode, with the ion-spray voltage set at −4,000 V, curtain gas at 35 (a.u.), source gas 1 and gas 2 at 50 and 40 (a.u.), respectively, and source temperature at 650°C. Data was acquired and processed using Multiquant software. Peak areas of individual metabolites were normalized against the peak areas of 13C1-Lactate and then normalized against the total protein concentration of each sample measured by the BCA assay.

Western Blot

After the animals were sacrificed, the injected gastrocnemius was quickly excised on ice. The gastrocnemius muscle tissue was stored at −80°C and the protein in whole gastrocnemius muscle tissue was extracted later using the following protocol: approximately 100 mg of the whole gastrocnemius muscle of each group ($n = 10$) was homogenized in ice-cold RIPA buffer and then centrifuged at 12,000 RPM for 30 min at 4°C. The supernatant's protein concentration was determined by BCA assay (Thermo): supernatant and gradient-diluted protein standards were placed in a 96-well plate with two duplicates and then mixed with the kit's A + B solution. After a 30 min incubation at 37°C and cooling to room temperature, the resultant purple color is measured using a spectrometer at 562 nm. In this way, the standard curve was determined, and the protein concentration could be calculated by application of the standard curve. The supernatant was finally trimmed by PBS for western blotting analysis.

About 40–50 μg protein for each lane was separated on a 10/12% SDS–PAGE gel and transferred onto a PVDF membrane. Then, blocked with 5% skimmed milk for 30–60 min. Antibodies used for western blotting were Anti-Rabbit Secondary Antibody (31,460, Invitrogen), Anti-Mouse Secondary Antibody (31,460, S0002), GAPDH (AF7021, Affinity), GPR81 (PA5-75664, Invitrogen), P-CREB (AF3189, Affinity), P-CREB (AB32096, Abcam), CREB (AF6188, Affinity), CREB (AB32515, Abcam), sterol regulatory element-binding protein 1c (SREBP-1C; AF4728, Affinity), PPAR-γ (AF6284, Affinity), P-HSL (AF2206, Beyotime), CS (DF13222, Affinity), P-ACC (Abcam, ab68191), acetyl-CoA carboxylase (ACC; CST, 3676 s), hormone-sensitive lipase (HSL; AF6403, Affinity), PKA (AF7746, Affinity), P-PKA (AF7246, Affinity), adipose triglyceride lipase (ATGL; AB207799, Abcam), P-ATGL (AB135093, Abcam), FASN (CST, 31,805), and β-tubulin (AB179513, Abcam). Blots were developed using Western Lightning ECL (Affinity). All the bands were analyzed with Image J. GAPDH and β-tubulin were used for the normalization of each protein to ensure the loading of equal quantities of protein.

Statistical Analysis

Data is presented as mean ± SD. The comparison between the means of two groups was assessed by the independent-samples *t*-test. The comparison among the multiple groups was assessed by one-way ANOVA. We assigned * for values below 0.05. Statistical graphs and statistical analyses were carried out using Prism 8 (GraphPad).

RESULTS

The Effect of Acute Intramuscular Lactate Injection on the Injected Gastrocnemius Lactate Concentration

The lactate concentration of gastrocnemius following acute intramuscular administration of lactate is shown in **Figure 1**. The muscle lactate concentration after PBS injection is 2.86 ± 0.22 μmol/g

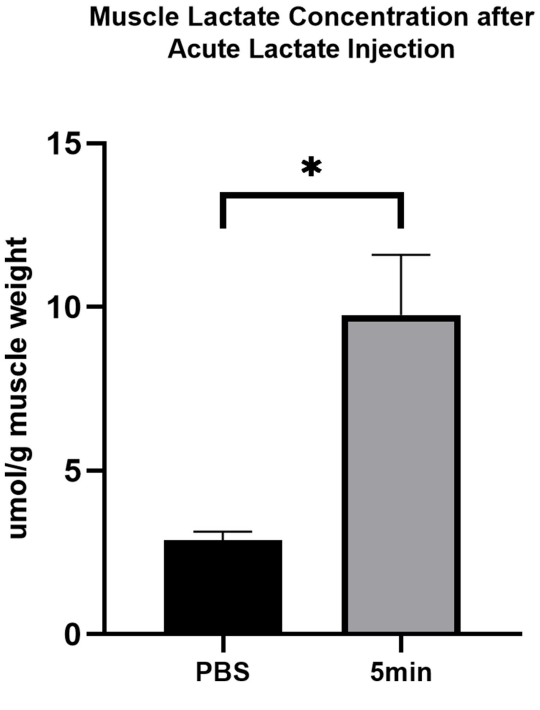

FIGURE 1 | The effect of acute intramuscular lactate injection on the injected gastrocnemius lactate concentration. The muscle lactate concentration was measured 5 min after PBS and lactate injection. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples *t*-test. $^{*}p < 0.05$ vs. 0 min.

muscle weight. Lactate level reached 9.7586 ± 1.49 μmol/g muscle weight at 5 min after acute intramuscular lactate injection ($p < 0.05$).

The Time-Dependent Effects of Acute Intramuscular Lactate Injection on Blood Lactate Concentration

The blood lactate concentration in mice following acute intramuscular administration of lactate is shown in **Figure 2**. The average resting blood lactate concentration of mice is 1.6 ± 0.1 mmol/L. Lactate level significantly increased after acute intramuscular lactate injection and reached peak value (3.8 ± 0.4 mmol/L) at 5 min (0 vs. 5 min; $p < 0.05$) and returned to baseline at 20 min.

The Time-Dependent Effects of Acute Lactate and Forskolin Injection on the cAMP-PKA Pathway

As shown in **Figure 3**, the ratios of P-PKA/PKA (Thr198) and P-CREB/CREB (Ser133) in gastrocnemius decreased after acute lactate injection, while acute forskolin injection upregulated the ratios of P-PKA/PKA and P-CREB/CREB. Furthermore, the effects of acute lactate treatment were most obvious 30 min after the injection, and the effects of acute forskolin treatment were most obvious 15 min after the injection.

The Blocking Effects of Acute Forskolin Treatment on Acute Lactate Injection Induced Inhibition of cAMP-PKA Pathway

As shown in **Figure 4**, we also validated forskolin's blocking effect on lactate-induced inhibition of cAMP-PKA pathway.

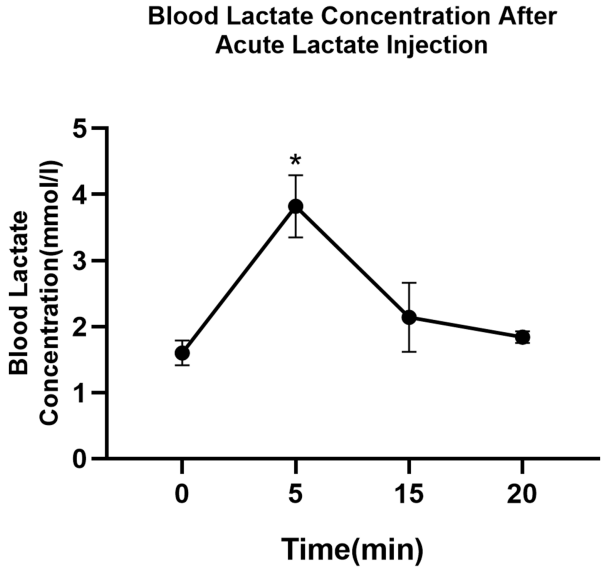

FIGURE 2 | The time-dependent effects of intramuscular lactate-injection on blood lactate level. The blood lactate was determined at rest and 5, 10, 15, and 20 min after the injection. The data are presented as the mean ± SD, and significant differences between two groups were analyzed with the independent-samples *t*-test. *$p < 0.05$ vs. 0 min.

Indicated by the ratios of P-CREB/CREB and P-PKA/PKA in AL-120, ALF, and AF-60 group, the effects of acute lactate injection on cAMP-PKA pathway were blocked by acute forskolin injection (values of p less than 0.05).

The Effects of Chronic Lactate and Forskolin Treatment on Intramuscular Triglyceride

As shown in **Figure 5A**, chronic lactate intervention significantly increased the abundance of triglycerides in the gastrocnemius ($p < 0.05$) compared to that of the chronic phosphate-buffered saline intervention (the solvent of lactate solution). Intramuscular triglycerides contents after chronic forskolin intervention were not downregulated compared to that of the chronic DMSO intervention (the solvent of forskolin solution). Moreover, the triglycerides accumulation effect of lactate was blocked by forskolin injection indicated by the results of the CLF group compared to that of the CL group ($p < 0.05$). These changes coincide with the results of oil red staining (**Figure 5B**).

The Effects of Chronic Lactate and Forskolin Treatment on Intramuscular GPR81 and the cAMp-PKA Pathway

As presented in **Figure 6**, chronic lactate treatment elevated the intramuscular expression of GPR81 in the CL group compared to that of the CP group ($p < 0.05$). The ratios of P-PKA/PKA (Ser133), the expression level of CREB and the phosphorylation level of CREB (Thr198) in the CL group were decreased after chronic lactate injection compared with the CP group ($p < 0.05$). Moreover, as indicated by the changing trend in the CLF group and CL group, the effects of lactate were blocked by forskolin injection (values of p less than 0.05). These results suggest that the chronic intramuscular injection of lactate successfully activated GPR81 to inhibit the cAMP-PKA pathway in skeletal muscle and forskolin is an effective inhibitor to block lactate's function on the cAMP-PKA pathway.

The Effects of Chronic Lactate and Forskolin Treatment on Intramuscular Lipid Metabolism-Related Proteins

To further investigate the mechanism of lactate-induced intramuscular triglyceride variation, we measured the expression levels of lipogenesis-related and lipolysis-related proteins in gastrocnemius after chronic injections. **Figure 7** provides that chronic lactate injection suppressed the phosphorylation of HSL (phospho S853), ATGL (phospho S406), and ACC (phospho S79), and upregulated the expression levels of SREBP and PPAR-γ (values of p less than 0.05). While chronic forskolin injection increased the phosphorylation levels of HSL, ATGL, and ACC, and downregulated the expression level of SREBP-1C (values of p less than 0.05) but not that of PPAR-γ. As indicated by the comparison between CLF group and CL group, lactate's effects on these proteins were blocked by forskolin injection (values of p less than 0.05). Moreover, our results indicated that the expression of FAS was not modulated after chronic lactate injection.

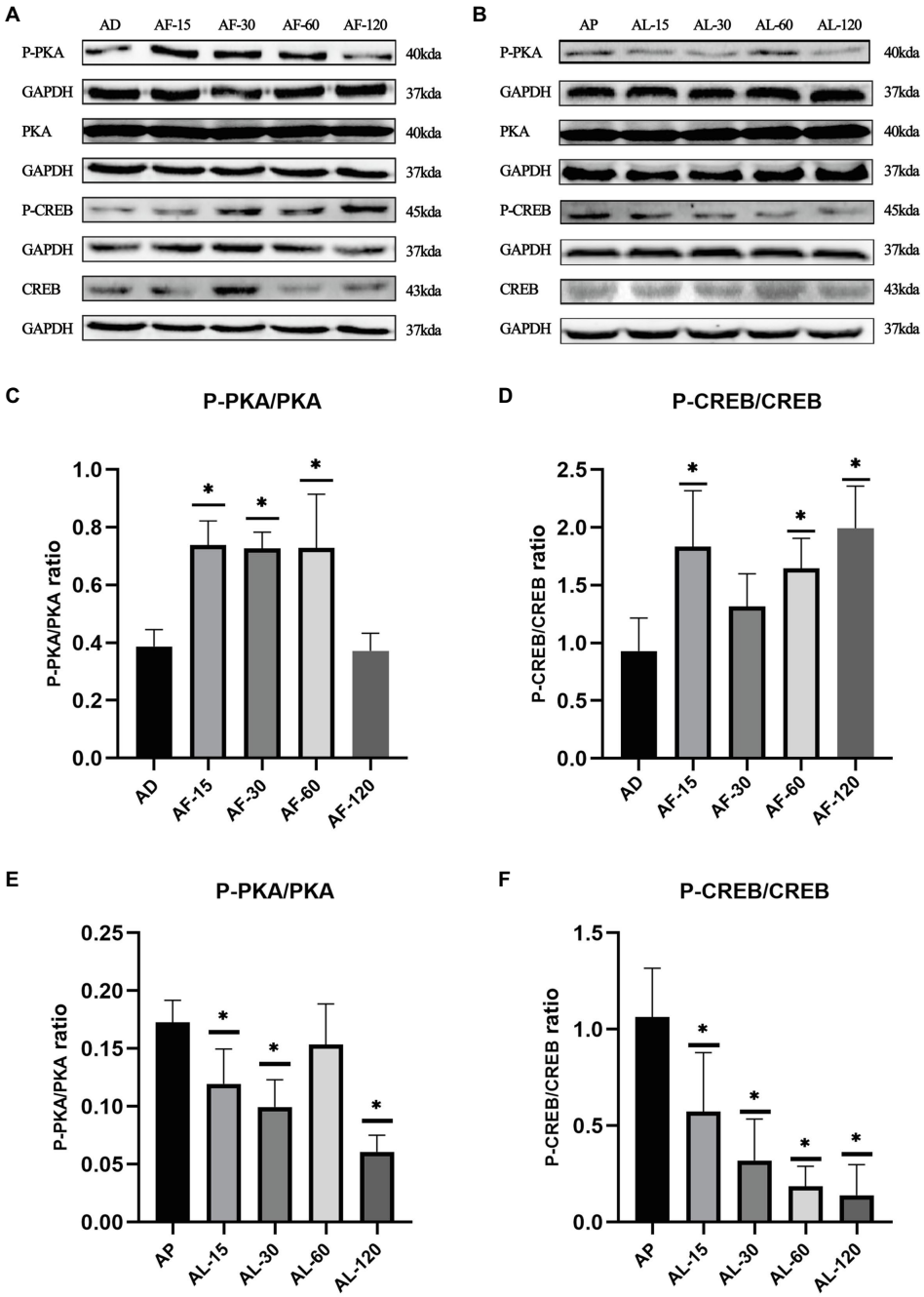

FIGURE 3 | The time-dependent effects of acute lactate and forskolin injection on the cAMP-PKA pathway. **(A)** Western blot analysis of cAMP-PKA pathway proteins after acute forskolin injection. **(B)** Western blot analysis of cAMP-PKA pathway proteins after acute lactate injection. **(C,D)** The ratios of P-PKA/PKA and P-CREB/CREB after acute forskolin injection. **(E,F)** The ratios of P-PKA/PKA and P-CREB/CREB after acute lactate injection. AL-15, AL-30, AL-60, and AL-120 represent 15, 30, 60, and 120 min after acute lactate injections. AF-15, AF-30, AF-60, and AF-120 represent 15, 30, 60, and 120 min after acute forskolin injections. AP and AD mean sacrificed after acute PBS and DMSO injection, respectively. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples *t*-test. $^{*}p < 0.05$ vs. AD and AP.

The Effects of Chronic Lactate and Forskolin Treatment on Intramuscular Mitochondria Content Biomarkers

Exercise-induced intramuscular triglyceride accumulation is always accompanied by mitochondria adaption to hydrolyze triglycerides more efficiently. To investigate the impact of lactate on mitochondria and its mechanism, the biomarkers of mitochondria content: CS expression level and its activity were investigated. As illustrated in **Figure 8**, the expression level of CS and its activity increased after chronic lactate

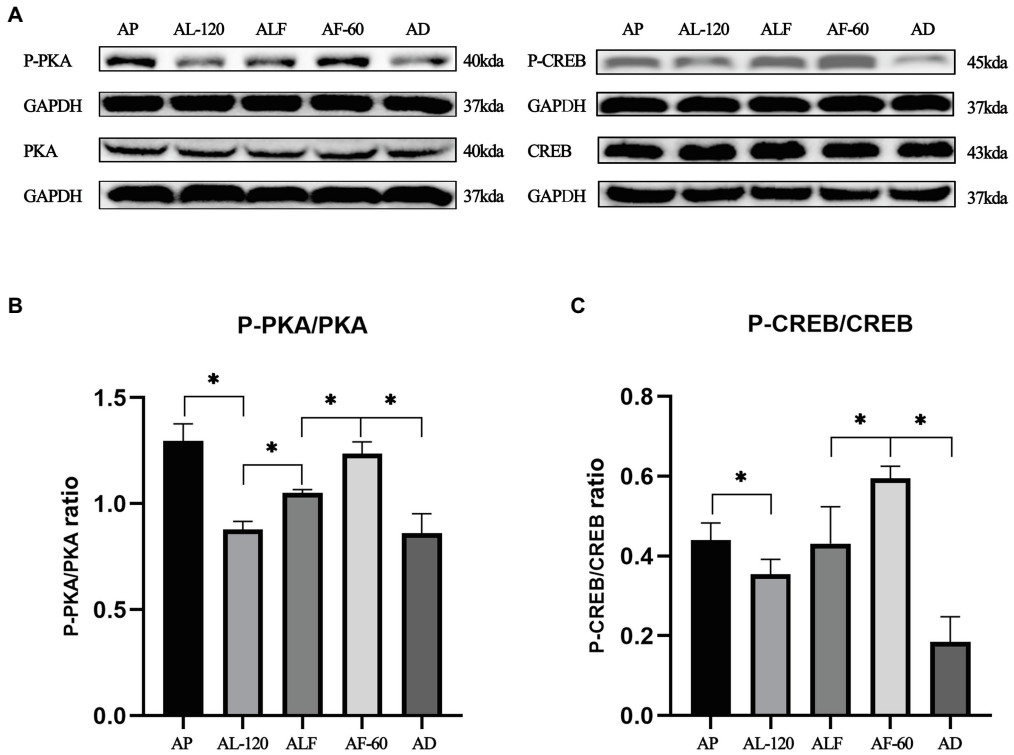

FIGURE 4 | The blocking effects of acute forskolin treatment on acute lactate injection induced inhibition of cAMP-PKA pathway. **(A)** Western blot analysis of cAMP-PKA pathway proteins after acute lactate and forskolin injections. **(B)** The ratios of P-PKA/PKA after acute lactate and forskolin injection. **(C)** The ratios of P-CREB/CREB after acute lactate and forskolin injection. AP and AD mean sacrificed after acute PBS and DMSO injection, respectively. ALF means sacrificed at 60 min after acute lactate and forskolin injection (60 min interval). AL-120: sacrificed 120 min after acute lactate injection. AF-60: sacrificed 60 min after acute forskolin injection. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences among the groups were analyzed with one-way ANOVA. $^{*}p < 0.05$.

injection ($p < 0.05$). Lactate's effect on the activity of CS was blocked by forskolin ($p < 0.05$). However, lactate-induced high expression of CS was not inhibited by the forskolin injection.

The Effects of Acute Lactate Injection on the Contents of Lactic Acid and TCA Related Metabolites in Mitochondria

The unexpected expression level of CS after chronic lactate treatment suggests other mechanisms might also contribute to the lactate-induced mitochondria content increase. Hence, we performed exploratory research on the potential metabolic mechanism of the lactate-induced mitochondria content increase. In the first place, to clarify whether injected lactate enters mitochondria and be oxidized there, we measured the contents of L-lactic acid and TCA-related metabolites in mitochondria after acute lactate injection. As shown in **Figure 9A**, the contents of L-lactic acid, succinic acid, L-malic acid, and oxalacetic acid after lactate injection were significantly increased compared to PBS injection (the value of p of each analysis is less than 0.05).

Colocalization of Mitochondria and LDHA

After clarified that injected lactate could be transported into mitochondria and oxidized there, we further validated the prerequisite for lactate oxidation in mitochondria. As shown in **Figure 9B**, LDHA exists in mitochondria of the gastrocnemius muscle, which provides a possibility for lactate oxidation in mitochondria and metabolism-related mechanisms of lactate-induced mitochondria content increase.

DISCUSSION

We have previously reported that lactate contributed to intramuscular triglyceride accumulation and mitochondria adaption in rats. However, the cellular/molecular mechanisms were unclear. The role of cAMP-PKA pathway in lactate-induced lipid accumulation in adipose tissue has been widely reported in previous studies (Sakurai et al., 2014). Moreover, CREB (downstream of cAMP-PKA pathway) is also reported to be associated with mitochondria biogenesis and content increase.

In the present study, we identified, for the first time, that cAMP-PKA pathway is involved in lactate-induced intramuscular triglyceride accumulation. We also explored possible mechanisms of increased mitochondria content after chronic lactate injection. The unique results ascertained in these trials were as follows: (1) chronic intramuscular lactate injection promoted lipid

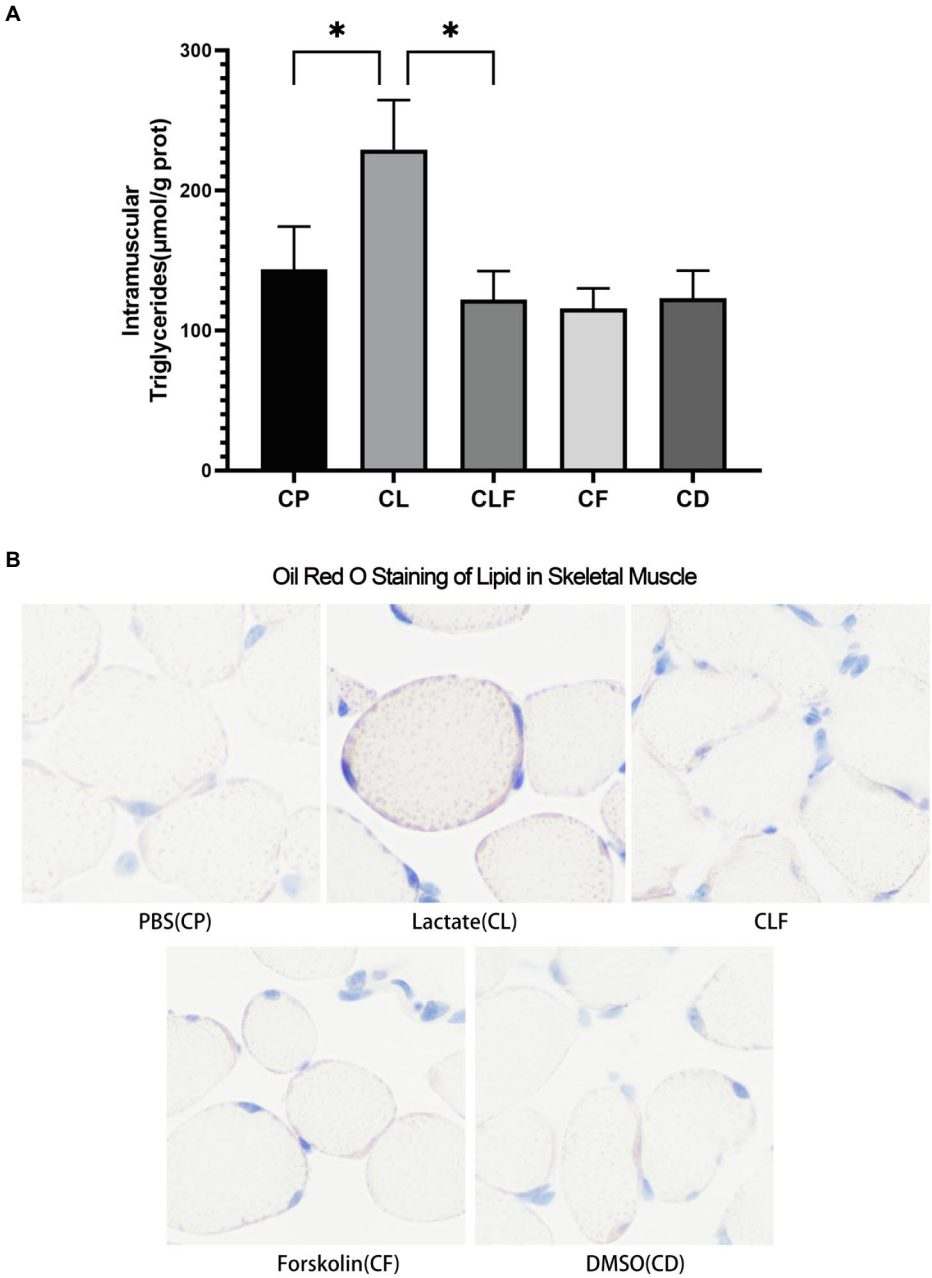

FIGURE 5 | Intramuscular triglyceride variation after chronic administration. **(A)** Intramuscular Triglycerides Abundance of Skeletal Muscle ($n = 6$). **(B)** The Oil Red O Staining of gastrocnemius of mice ($n = 5$). CP, chronic PBS treated group; CL, chronic lactate treated group; *CF*, chronic forskolin treated group; CD, chronic DMSO treated group; and CLF, chronic lactate and forskolin treated group. Other images can be found in the additional materials. Measurement data were expressed by mean ± SD, and significant differences among the groups were analyzed with one-way ANOVA. $^{*}p < 0.05$.

accumulation by suppressing lipolysis and stimulating lipogenesis. The suppressed lipolysis and stimulated lipogenesis were partially blocked by forskolin; (2) CS content and its activity increased after chronic intramuscular lactate injection, and the lactate-induced CS content change was not blocked by forskolin treatment; and (3) the contents of lactate-related metabolites in skeletal muscle mitochondria increased after acute lactate injection, and LHDA was also validated to exist in mitochondria. These results provide a possibility for metabolism-related mechanisms of lactate-induced mitochondria content increase.

High-intensity training is widely known to induce intramuscular lipid accumulation (van Loon and Goodpaster, 2006; Shaw et al., 2012). The adaptation of intramuscular triglyceride storage after exercise may benefit exercising tissue by supplying free fatty acids (Zacharewicz et al., 2018). However, the molecular mechanism underlying this adaptation is unknown.

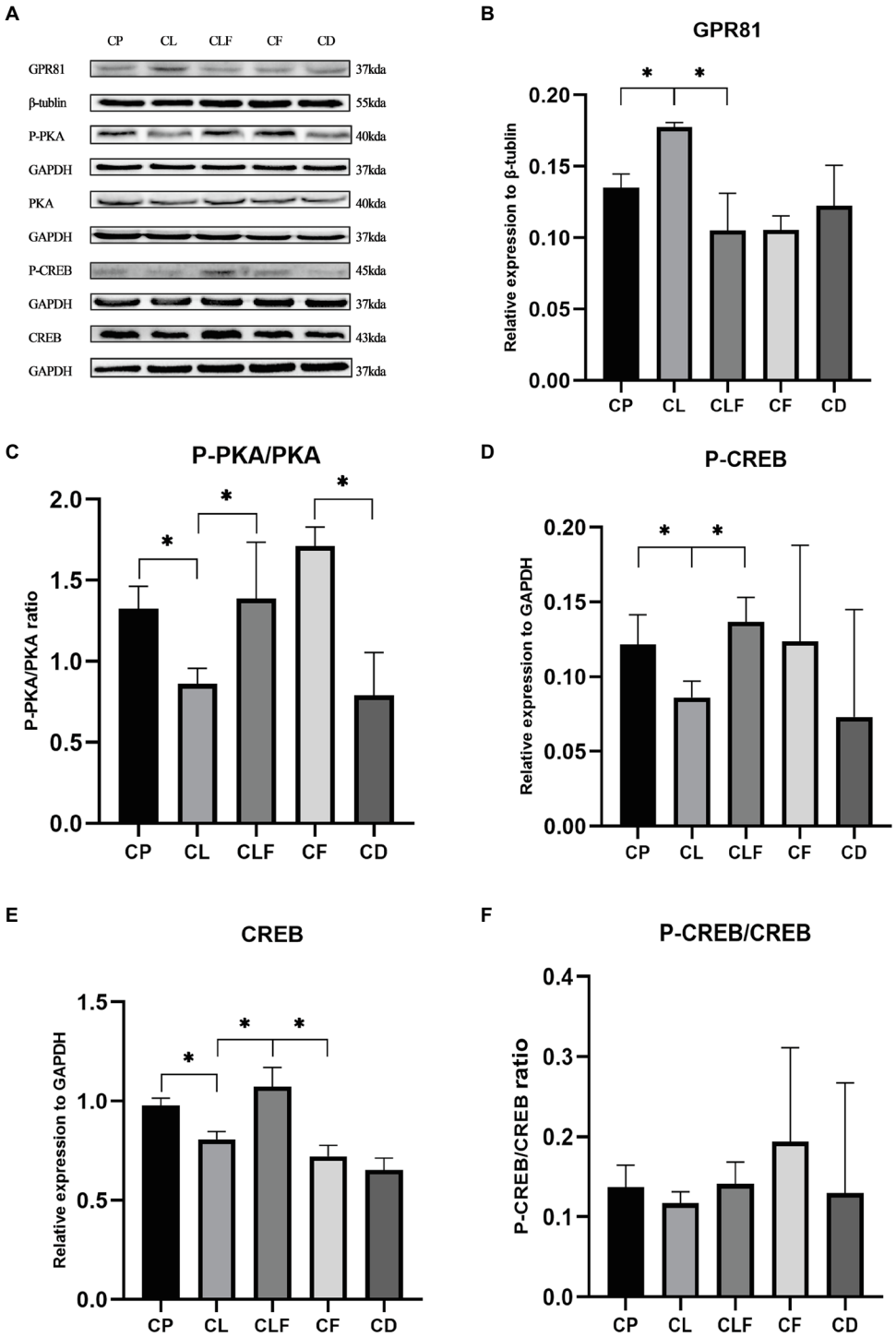

FIGURE 6 | Western blot analysis and relative fold protein expression of GPR81, P-CREB, CREB, and the ratios of P-PKA/PKA and P-CREB/CREB after chronic administration. **(A)** Western blot analysis of GPR81 and proteins involved in cAMP-PKA pathway. **(B)** Fold protein expression of GPR81. **(C)** The ratio of P-PKA/PKA. **(D)** Fold protein expression of P-CREB. **(E)** Fold protein expression of CREB. **(F)** The ratio of P-CREB/CREB. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences among the groups were analyzed with one-way ANOVA. $^{*}p < 0.05$.

Our prior studies have provided evidence that lactate might be one of the triggers (Zhou et al., 2021b). In that study, we have identified that after one-time high-intensity exercise, the average blood lactate concentration in rats was 5.08 ± 1.38 mmol/L. According to previous studies, using a certain dose [0.64 ml/kg lactate (0.25 M)] of lactic acid solution

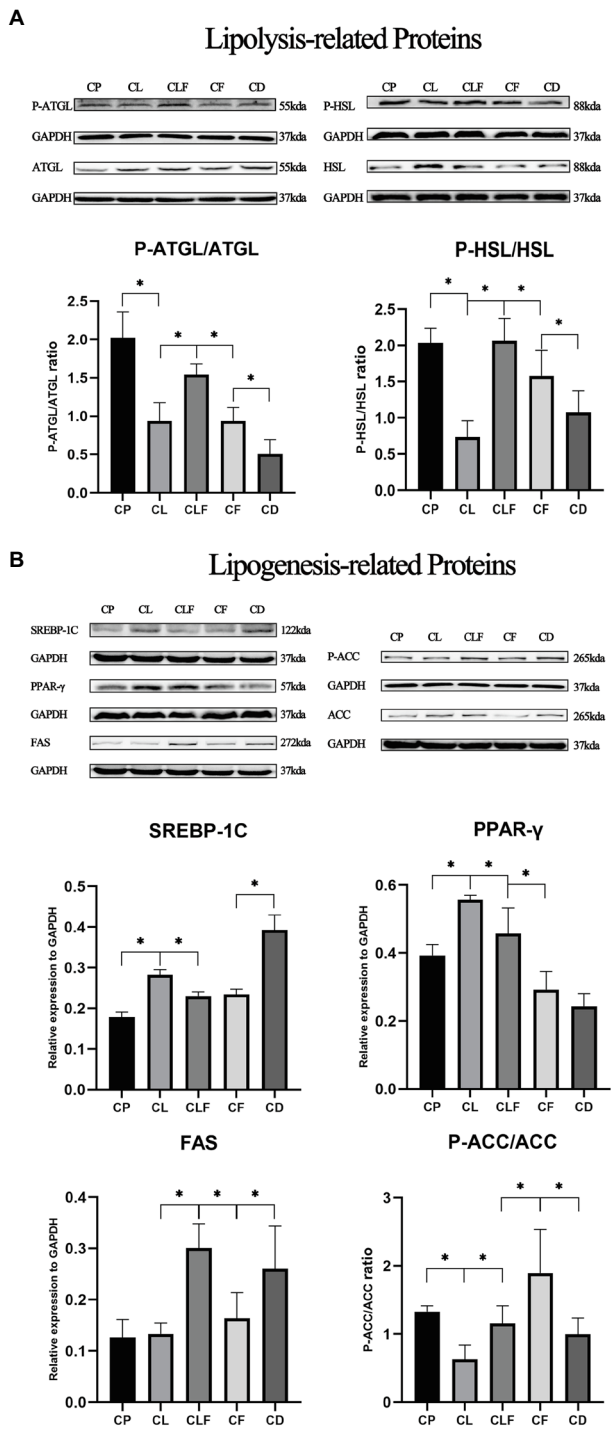

FIGURE 7 | The expression levels of lipid metabolism-related proteins after chronic lactate and forskolin injection. **(A)** Western blot analysis and relative fold protein expression of lipolysis-related proteins. **(B)** Western blot analysis and relative fold protein expression of lipogenesis-related proteins.
CP, chronic PBS treated group; CL, chronic lactate treated group; *CF*, chronic forskolin treated group; CD, chronic DMSO treated group; and CLF, chronic lactate and forskolin treated group. Relative expression levels were normalized to GAPDH. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences among the groups were analyzed with one-way ANOVA. $^{*}p < 0.05$.

injected into the gastrocnemius muscle of rodents can simulate exercise-induced lactate changes (Nikooie and Samaneh, 2016; Zhou et al., 2021b). Here, we tested the previously established injection protocol in mice and found that muscle lactate concentration reached 9.7586 ± 1.49 μmol/g muscle weight and the blood lactate level reached 3.8 ± 0.4 mmol/L after the injection, which is close to exercise-induced changes in blood lactate. Therefore, we used 0.25 M lactate solution in a dose of 0.64 ml/kg to mice's gastrocnemius to stimulate the transient elevation of systemic lactate levels after exercise.

The role of GPR81 and cAMP-PKA pathway in lactate-induced lipid accumulation has been widely reported in previous studies on adipose tissue. For example, lactate-induced suppression of lipolysis in explants of white adipose tissue (WAT) has been confirmed to depend on the presence of GPR81 (Sakurai et al., 2014), which is closely related to the inhibition of cAMP-PKA pathway (Brown et al., 2020). It is also illustrated that lactate activates the G protein-coupled receptor GPR81 in adipocytes and mediates antilipolytic effects through Gi-dependent inhibition of adenylyl cyclase (Ahmed et al., 2010). In contrast to lactate, forskolin has been long recognized as a classic activator of the cAMP-PKA pathway (Sakurai et al., 2014). Hence, in this study, forskolin was selected as the inhibitor of lactate to investigate whether lactate's effects on intramuscular triglyceride accumulation and mitochondria content are modulated by the cAMP-PKA pathway.

Before the chronic administration, we explicated the time-dependent effects of acute lactate and forskolin injections on the intramuscular cAMP-PKA pathway and the forskolin's inhibitory effects on lactate. Results showed that the ratios of P-PKA/PKA and P-CREB/CREB increased after acute forskolin injection, while acute lactate injection suppressed the expression of these proteins. Forskolin's inhibitory effect on lactate was validated by the expression levels of ALF group. Furthermore, lactate's inhibitory effects on the cAMP-PKA pathway were most obvious at 30 min after injection. Forskolin's activation effects on the cAMP-PKA pathway were most obvious at 15 min after the injection. Hence, we selected an interval of 15 min between lactate injection and forskolin injection in chronic administration to ensure the forskolin's inhibitory effects on lactate's function (**Figure 10**).

Lactate's role on lipid accumulation in adipocytes has been demonstrated (Gold et al., 1963; Bjorntorp, 1965; Houghton et al., 1971; Boyd et al., 1974; De Pergola et al., 1989; Cai et al., 2008), and the mechanism of it might be lactate activates its receptor GPR81 and then inhibit the cAMP-PKA pathway to inhibit lipolysis (Sakurai et al., 2014). Whether lactate results in a similar process in skeletal muscle is unclear. In this study, the abundance of triglyceride in the gastrocnemius increased significantly after lactate intervention compared to that of the chronic phosphate-buffered saline intervention. This effect of lactate was significantly blocked by forskolin injection, suggesting lactate-induced intramuscular triglyceride accumulation is regulated by the cAMP-PKA pathway. The results of the ratios of P-PKA/PKA and P-CREB/CREB also confirmed this point of view. However, one unanticipated finding was that there was no significant difference between the intramuscular

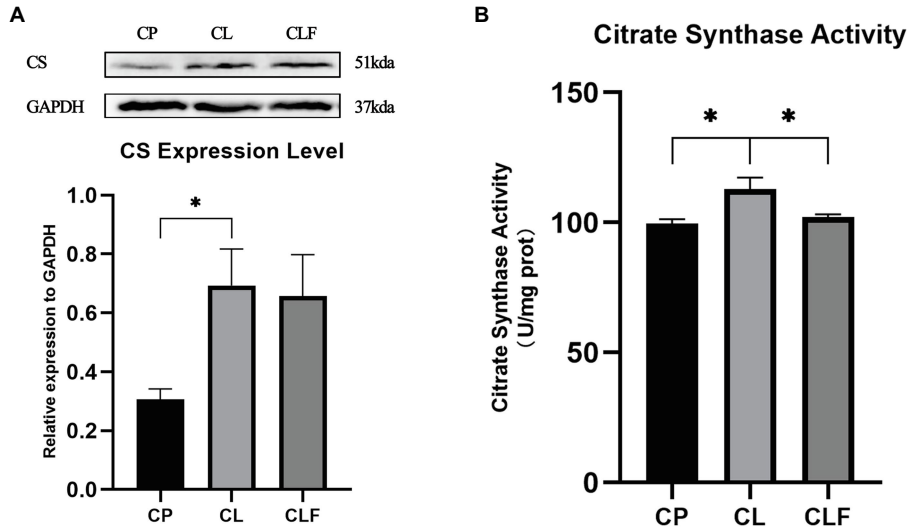

FIGURE 8 | The activity and expression level of citrate synthase (CS). **(A)** The expression level of CS in gastrocnemius after chronic administration ($n = 8$). **(B)** The activity of CS after chronic administration. CP, chronic PBS treated group; CL, chronic lactate treated group; and *CF*, chronic forskolin treated group. Relative expression levels were normalized to GAPDH. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences among the groups were analyzed with one-way ANOVA. $^{*}p < 0.05$.

triglyceride contents in the chronic DMSO treatment group and the forskolin treatment group. This result may be explained by the hypothesis that a self-protection mechanism might be activated to prevent fatty acids from being hydrolyzed (Zhou et al., 2021a) when the intramuscular abundance of triglycerides was not elevated by external intervention, and further investigation is suggested to validate it.

Previous studies have found that lactate-induced intramuscular triglycerides accumulation coincides with the inhibited lipolysis and promoted lipogenesis (Zhou et al., 2021b). Here, to further investigate the role of the cAMP-PKA pathway in lactate-induced intramuscular triglyceride accumulation, we measured the expression levels of lipolysis-related proteins (P-HSL/HSL and P-ATGL/ATGL) and lipogenesis-related proteins (SREBP, PPAR-γ, FAS, and P-ACC/ACC) in gastrocnemius after chronic administration. ATGL plays a key role in lipid droplet/adiposome degradation (Smirnova et al., 2006). ATGL catalyzes the initial step in triglyceride hydrolysis in adipocytes, and it is also reported to regulate triglyceride hydrolysis in muscle recently (Smirnova et al., 2006; Zhao et al., 2020). HSL is believed to play a regulatory role in initiating the degradation of intramuscular triacylglycerol in skeletal muscle (Watt and Spriet, 2004). It is known to be the rate-limiting enzyme for hydrolysis of triacylglycerol in adipocytes at first, and also be proved to regulate the intramyocellular hydrolysis of triacylglycerol to diacylglycerol and further on to monoacylglycerol (Donsmark et al., 2005). In this study, the phosphorylation of HSL and ATGL was suppressed after chronic lactate injection, and this variation was successfully blocked by forskolin injection. However, the phosphorylation levels of HSL and ATGL in CLF group were higher than those in the *CF* group. A possible explanation for this might be that repetitive injection stimulation induced chronic stress and led to the activation of HSL and ATGL. Further study is needed to validate this hypothesis since previous study only investigated the expression of mRNA in HSL and ATGL under chronic stress (Mu et al., 2019). Meanwhile, it is also indicated that other mechanisms might contribute to the lactate-induced intramuscular triglyceride accumulation. Hence, our results of lipolysis-related proteins suggest, to a great extent, the lactate-induced intramuscular triglyceride accumulation is achieved by inhibition of lipolysis, and this process is regulated by the cAMP-PKA pathway.

Correspondingly, lipogenesis-related proteins were also investigated in this study. SREBP-1C is a bound transcription factor that activates genes encoding enzymes required for the synthesis of cholesterol and unsaturated fatty acids (Debose-Boyd and Ye, 2018). The cAMP/PKA pathway is recognized to negatively regulate SREBP-1C expression at the transcriptional and post-translational levels (Zhou et al., 2007). Here, the expression of SREBP-1C was promoted after chronic lactate injection and this variation was inhibited by forskolin injection, though no difference was observed between the SREBP expression levels in the CLF group and *CF* group. Therefore, cAMP-PKA-SREBP-1C pathway might contribute to lactate-induced intramuscular triglyceride accumulation, and other mechanisms besides this pathway might also play a role. PPAR-γ is also an important lipogenesis-related protein (Choi et al., 2011; Koves et al., 2013). According to our results, PPAR-γ might contribute to lactate-induced intramuscular triglyceride accumulation and this process might be regulated by the cAMP-PKA pathway, but no significant difference between CD and *CF* group suggests other mechanisms might also regulate PPAR-γ. FAS is crucial for the synthesis of saturated long-chain fatty acids and it is also reported to be regulated by SREBP-1C (Semenkovich, 1997; Griffin et al., 2007; Roder et al., 2007; Choi et al., 2008). However, no significant difference

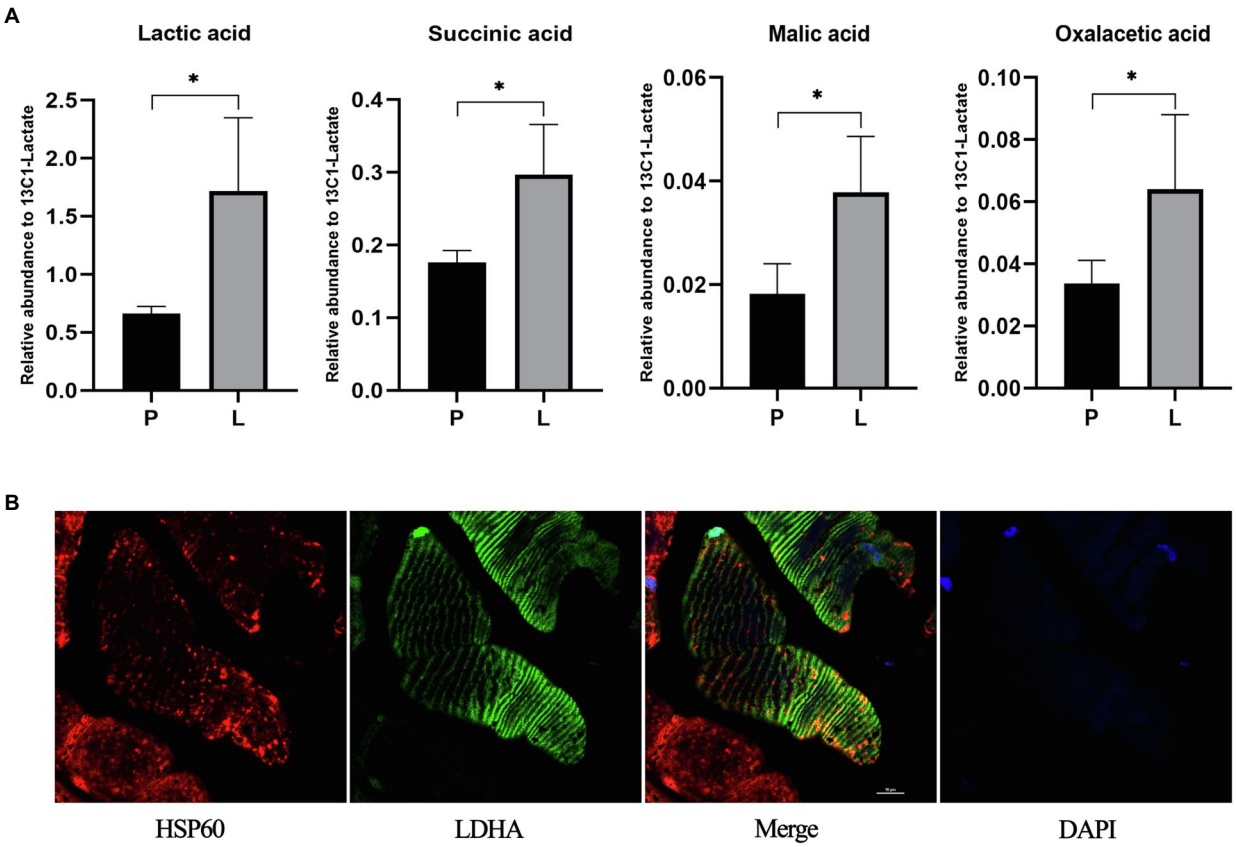

FIGURE 9 | The contents of lactic acid and TCA related metabolites in mitochondria after acute lactate injection and the colocalization of mitochondria and LDHA. **(A)** The relative abundance of Lactic acid, Succinic acid, Malic acid, and Oxalacetic acid in mitochondria. P: after acute PBS injection ($n = 3$); L: after acute lactate injection ($n = 3$). The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. $^{*}p < 0.05$. **(B)** Confocal laser scanning microscopic imaging of immunolabeled LDH (green), and HSP60 (red). Antibodies against HSP60 were used as a label of the mitochondria.

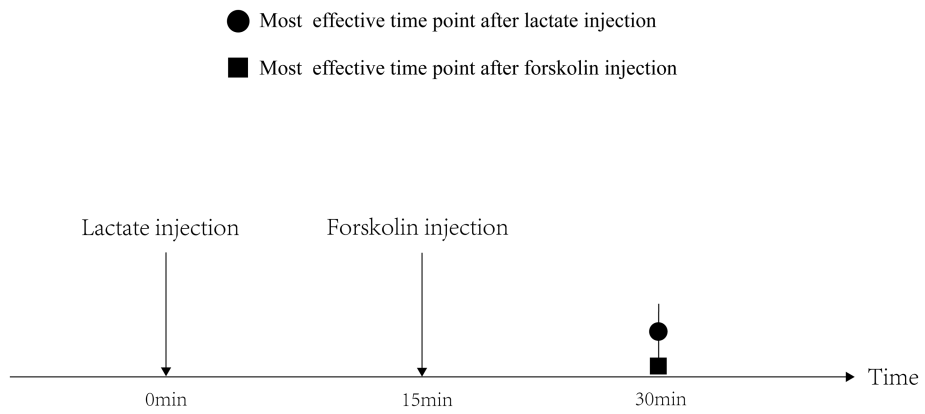

FIGURE 10 | Effectivity of lactate and forskolin injection on cAMP-PKA pathway.

was observed after lactate injection and the expression levels of FAS in CLF, *CF*, and CD groups are confusing. This result may be explained by the fact that FAS is also regulated by many hormones (Donkin et al., 1996; Najjar et al., 2005; Xu et al., 2005), and further study is needed to explicate it. ACC catalyzes the formation of malonyl-CoA, an essential substrate for fatty acid synthesis in lipogenic tissues and a key regulatory molecule in muscle, brain, and other tissues

(Wakil, 1958; Brownsey et al., 2006). The phosphorylation of ACC changes the ACC from the active form of a large linear polymer to the inactive form (Cho et al., 2010), therefore, the low phosphorylation level of ACC represents promoted lipogenesis (Underwood et al., 2007; Labrie et al., 2015; Sun et al., 2019). In this study, the phosphorylation of ACC was downregulated after chronic lactate injection and this variation was blocked by forskolin injection. The above results of lipogenesis-related proteins imply that the lactate-induced intramuscular triglyceride accumulation might be achieved by promoting the expression of lipogenesis-related proteins, and mechanisms besides cAMP-PKA pathway might also play a prominent role in this process.

Previous studies have validated that intramuscular triglyceride accumulation is accompanied by mitochondria biogenesis and content increase in skeletal muscle (Koves et al., 2013). Our recent research also verified that lactate injection could increase the expression levels of mitochondria content biomarkers, but the mechanism remains unknown. Here, we implemented a tentative research to explore the metabolism-related possibility of the intramuscular mitochondria content increase after lactate injection. CS content and its activity have long been recognized as biomarkers of mitochondrial content (Nederlof et al., 2017; Gil et al., 2019; Wang et al., 2020). According to our results, the activity of CS was promoted after chronic lactate injection and this variation was inhibited by forskolin injection. However, the changes in CS activity were of low magnitude (CL is 13.3% higher than CP, and CLF is 9.5% lower than CL). The expression level of CS was also elevated after lactate injection ($p < 0.05$), and this effect was not inhibited by forskolin injection as indicated by the expression levels of the CL and CLF groups. This suggests the expression of CS might not be regulated by the cAMP-PKA signaling pathway, and other mechanisms (e.g., metabolic mechanism) of the lactate-induced mitochondria content increase might exist.

Hence, we performed explorative research for the potential mechanisms. We found that the contents of lactate-related metabolites in the mitochondria of skeletal muscle increased after acute lactate injection, and LHDA was also validated to exist in mitochondria in this study. These results provide a possibility for metabolism-related mechanisms of lactate-induced mitochondria content increase: lactate could enter mitochondria and be oxidized there, which might induce an adaptive increase of mitochondria content.

CREB has long been recognized as a nuclear transcription factor (Yan et al., 2016). In addition to its functions in the nucleus, it is also imported into the mitochondria and activates transcription of mitochondria proteins (Lee et al., 2005; Wu et al., 2006; Bergman and Ben-Shachar, 2016). In this study, lactate injection decreased phosphorylation of CREB, which might suppress mitochondrial transcription. This seemingly contradictory phenomenon might actually indicate the presence of the metabolism-related mechanisms, and the metabolism-induced mitochondria adaption might be stronger than the effects of CREB.

Composed of mMCT1, CD147 (basigin), mLDH, and cytochrome oxidase (COX), mLOC plays a key role in the oxidation of lactate in mitochondria (Hashimoto and Brooks, 2008). MCT4 is also always mentioned together with MCT1 as important lactate transporters in muscle (Bonen, 2001). Hence, mLOC could be a unique complex that warrants further attention to test our hypothesis.

In addition to metabolism-related mechanisms, the possibility of other potential mechanisms of lactate-induced mitochondrial biogenesis might exist. It has been reported that lactate could stimulate ROS generation, which activates transcription factors including not only CREB, but also factor-kappaB, nuclear factor erythroid-2, and nuclear respiratory factor-2 (Hashimoto and Brooks, 2008). Hence, transcription factors besides CREB might also contribute to lactate-induced mitochondria biogenesis and content increase, this seems to be a possible explanation for the results that forskolin (CREB activator) failed to fully suppress lactate-induced mitochondria biogenesis and content. In this study, the activity and expression level of CREB was inhibited, indicating the effects of GPR81-cAMP signaling pathway might be stronger than that of ROS. However, with very little evidence, this is only a hypothesis that needs to be confirmed by future studies.

In conclusion, based on our findings from previous and current studies, we propose that the lactate-induced intramuscular triglyceride accumulation is achieved by inhibition of lipolysis, and this process is regulated by the cAMP-PKA pathway. Suppressed lipogenesis also contributes to the lactate-induced triglyceride accumulation, and this process might be regulated by the cAMP-PKA pathway. Lactate injection might increase mitochondria content and cAMP-PKA pathway might have a limited contribution to it, while lactate-related metabolism process and various ROS-related transcription factors might play a role.

LIMITATION

Relative to the total volume of a mouse gastrocnemius, the volume of injection into the gastrocnemius was high in this study. This might mechanically disrupt the muscle bed and influence the experimental results. Additionally, this study only implemented a tentative research to explore the metabolism-related possibility of the intramuscular mitochondria content increase after lactate injection. Future study is needed to validate our hypothesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: https://figshare.com/s/da4fa6da5efaccbcb7ad.

ETHICS STATEMENT

The animal study was reviewed and approved by Medical Ethics Committee of Sichuan University.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Exercise-Induced Lactate Release Mediates Mitochondrial Biogenesis in the Hippocampus of Mice *via* Monocarboxylate Transporters

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Regular exercise training induces mitochondrial biogenesis in the brain *via* activation of peroxisome proliferator-activated receptor gamma-coactivator 1α (PGC-1α). However, it remains unclear whether a single bout of exercise would increase mitochondrial biogenesis in the brain. Therefore, we first investigated whether mitochondrial biogenesis in the hippocampus is affected by a single bout of exercise in mice. A single bout of high-intensity exercise, but not low- or moderate-intensity, increased hippocampal PGC-1α mRNA and mitochondrial DNA (mtDNA) copy number at 12 and 48 h. These results depended on exercise intensity, and blood lactate levels observed immediately after exercise. As lactate induces mitochondrial biogenesis in the brain, we examined the effects of acute lactate administration on blood and hippocampal extracellular lactate concentration by *in vivo* microdialysis. Intraperitoneal (I.P.) lactate injection increased hippocampal extracellular lactate concentration to the same as blood lactate level, promoting PGC-1α mRNA expression in the hippocampus. However, this was suppressed by administering UK5099, a lactate transporter inhibitor, before lactate injection. I.P. UK5099 administration did not affect running performance and blood lactate concentration immediately after exercise but attenuated exercise-induced hippocampal PGC-1α mRNA and mtDNA copy number. In addition, hippocampal monocarboxylate transporters (MCT)1, MCT2, and brain-derived neurotrophic factor (BDNF) mRNA expression, except MCT4, also increased after high-intensity exercise, which was abolished by UK5099 administration. Further, injection of 1,4-dideoxy-1,4-imino-D-arabinitol (glycogen phosphorylase inhibitor) into the hippocampus before high-intensity exercise suppressed glycogen consumption during exercise, but hippocampal lactate, PGC-1α, MCT1, and MCT2 mRNA concentrations were not altered after exercise. These results indicate that the increased blood lactate released from skeletal muscle may induce hippocampal mitochondrial biogenesis and BDNF expression by inducing MCT expression in mice, especially during short-term high-intensity exercise. Thus, a single bout of exercise above the lactate threshold could provide an effective strategy for increasing mitochondrial biogenesis in the hippocampus.

Keywords: exercise, hippocampus, peroxisome proliferator-activated receptor gamma-coactivator 1α, mitochondrial biogenesis, lactate, microdialysis

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INTRODUCTION

Mitochondrial dysfunction causes neurodegenerative diseases, such as Alzheimer's disease, and Parkinson's disease and metabolic diseases, such as type 2 diabetes; it is caused by physiological deterioration owing to aging and lack of exercise (Short et al., 2005; Sutherland et al., 2009; Safdar et al., 2011; Zhang et al., 2012; Rodriguez-Martinez et al., 2013). Mitochondrial biogenesis, the formation of new mitochondria in cells, is vital for mitochondrial function in various tissues. Endurance exercise training induces brain and skeletal muscle mitochondrial biogenesis (Steiner et al., 2011; Halling et al., 2019).

Peroxisome proliferator-activated receptor gamma-coactivator 1-α (PGC-1α) is the master regulator of mitochondrial biogenesis in various cell types (Sutherland et al., 2009; Wareski et al., 2009; Lezi et al., 2013). Eight weeks of treadmill exercise training induces an increase in PGC-1α mRNA expression in skeletal muscle and brain regions, including the cortex and hippocampus, with a concomitant increase in mitochondrial DNA (mtDNA) copy number, enhancing exercise performance (Steiner et al., 2011). Training adaptation reflects the accumulation of the beneficial physiological functions produced from single bouts of exercise; thus, to produce a better exercise training strategy, it is essential to understand the beneficial effects of a single bout of exercise and elucidate the mechanism underlying exercise. However, it remains unclear whether a single bout of exercise would increase mitochondrial biogenesis in the brain.

Research indicates that a single bout of exercise (low, moderate, and high intensity) increases the transcriptional regulators of mitochondrial biogenesis [PGC-1α, mitochondrial transcription factor A (TFAM), and nuclear respiratory factor1 (NRF1) mRNA expression] in mice and human skeletal muscle (Safdar et al., 2009; Little et al., 2010; Tadaishi et al., 2011; Saleem and Hood, 2013; Gidlund et al., 2015), proportional to the exercise intensity used (Ikeda et al., 2008). These results suggest that even a single bout of exercise enhances mitochondrial biogenesis in skeletal muscle, and the extent of its enhancement depends on the intensity of exercise performed. On the other hand, it is unclear whether hippocampal mitochondrial biogenesis is also affected by a single bout of exercise and whether its effect, if any, depends on exercise intensity.

Skeletal muscles release lactate into the blood during exercise; blood lactate concentrations range from ~3 mM at rest to ~10 mM in mice following high-intensity exercises, which is above the lactate threshold (LT; Hu et al., 2021). LT is the time point when blood lactate concentrations start to rise from the resting level if the subjects continue to exercise with increasing exercise intensity. This time point is thought to express the increased usage of both muscle glycogen and fast muscle fibers (Billat et al., 2005). Lactate was thought to be only a waste product derived from glycolysis metabolism. However, recent studies revealed that circulating blood lactate serves as energy substrates for the skeletal muscle (Takahashi et al., 2020) and enhances skeletal muscle mitochondrial biogenesis (Rowe et al., 2013; Kitaoka et al., 2016). Additionally, lactate crosses the blood–brain barrier (BBB) through monocarboxylate transporters (MCT) in brain cells (Riske et al., 2017; El Hayek et al., 2019) and supplies the energy substrates to neurons (Kobayashi et al., 2019; Lev-Vachnish et al., 2019). For example, 6-week high-intensity interval training (HIIT) increased hippocampal lactate concentration and enhanced mitochondrial biogenesis (Hu et al., 2021). In addition, HIIT also increased hippocampal brain-derived neurotrophic factor (BDNF) expression and protein, which is a necessary regulator for enhancing mitochondrial quality control, and maintaining neuronal function and survival (Freitas et al., 2018; Hu et al., 2021; Okamoto et al., 2021).

Exogenous intraperitoneally (i.p.) or orally administered lactate works the same way as endogenous lactate released from skeletal muscle during exercise. Lactate administrated chronically to mice functioned as a signal molecule that promoted adult hippocampal neurogenesis (Lev-Vachnish et al., 2019). Acute and chronic peripheral lactate administration increased extracellular lactate concentrations in hippocampal tissue, which produced antidepressant-like effects in mice (Carrard et al., 2018). Lactate administered to hippocampal cells *in vitro* enhanced ATP levels, PGC-1α, BDNF protein level, mtDNA copy number, and potentiated mitochondrial function (Hu et al., 2021). We hypothesized that blood lactate increased by high-intensity exercise could promote PGC-1α mRNA expression in the hippocampus and trigger hippocampal mitochondrial biogenesis.

Lactate derived from brain glycogen is critical for neurons. In brief, lactate generated by glycogen degradation in astrocytes is transferred to the neurons *via* MCTs and acts as energy sources for neurons, helping maintain their function; this system is known as the astrocyte-neuronal lactate shuttle (ANLS; Brooks, 2002). The ANLS has been referred to play a key role in lactate transport and neuronal activity (Pellerin et al., 1998), which is involved in the hippocampal function, such as long-term potentiation and long-term memory as well as endurance exercise capacity (Newman et al., 2011; Suzuki et al., 2011; Lev-Vachnish et al., 2019). Matsui et al. found that a bout of acute moderate-intensity exercise increased hippocampal lactate and ATP levels and decreased brain glycogen concentrations, which was abolished by 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) administration, a glycogenolysis inhibitor, and diminished endurance exercise capacity (Matsui et al., 2017). We thus hypothesized that glycogenolysis inhibition during exercise might also attenuate exercise-induced PGC-1α and MCTs mRNA expression in the hippocampus. Furthermore, intracerebral DAB administration decreased neuron lactate supply from astrocytes and was deleterious to rat's cognitive function (Suzuki et al., 2011; Matsui et al., 2017). These results suggest that lactate derived from brain glycogenolysis is vital for maintaining brain functions. However, no reports exist on whether lactate derived from brain glycogenolysis contributes to mitochondrial biogenesis after high-intensity exercise.

This study assessed the effects of a single bout of low-, moderate-, or high-intensity treadmill exercise on PGC-1α mRNA expression and mtDNA copy number in the mouse hippocampus. Next, we examined whether exogenous lactate administration increased hippocampal extracellular lactate concentration and mimicked the effects of high-intensity exercise.

Then, we examined if exogenous lactate or UK5099, a lactate transporter inhibitor, administered before high-intensity exercise alters mouse hippocampus PGC-1α mRNA expression and mtDNA copy number as well as BDNF expression. Finally, we examined whether pre-exercise intra-hippocampal injection of DAB affected the mouse hippocampal PGC-1α and MCTs mRNA expression following high-intensity exercise.

MATERIALS AND METHODS

Ethical Approval

Animal use and procedures followed the National Institute of Health guideline and were approved by the Animal Care and Use Committee of Nippon Medical School (approval no. 30-030). Furthermore, we exerted all efforts to minimize animal pain and discomfort.

Animals

Eight-week-old male ICR mice (weight: 36–38 g; Sankyo Lab, Tokyo, Japan) were used in this study. All mice were housed (5 per cage) in standard transparent mouse cages (29 × 18 × 13 cm) and provided *ad libitum* access to standard chow (MF; Oriental Yeast Co, Ltd., Tokyo, Japan) and water. Mice selected for surgery were individually housed in the same cages. Room temperature was maintained at 22–24°C with 50% humidity under a 12 h light/dark cycle (lights on: 08:00–20:00). Following each experiment, all mice were killed by decapitation using a guillotine without anesthesia, which was the most humane method, taking both animal welfare and data quality into consideration (Pierozan et al., 2017).

Treadmill Exercise Protocol

All mice were subjected to 15 min of treadmill running at a treadmill running speed of 10–15 m/min for three consecutive days during the habituation period. After 3 days of rest following the treadmill habituation period, mice performed treadmill running at low, moderate, or high intensity. The protocol for a single bout of exercise at three intensities is shown in **Figure 1A**. In brief, low- and moderate-intensity exercise group mice were subjected to a single bout of running on a treadmill at speeds of 10 or 20 m/min, respectively, for 30 min.

High-intensity exercise consisted of intense intermittent running, including rest, on a treadmill with a gradual increase in speed determined according to the method of Lee et al. (2018) as follows: The mice ran for 1 min on a treadmill set at a treadmill speed of 20 m/min and then rested for 10 s, which was defined as one set. After the mice performed three sets at the same treadmill speed, the speed was increased to 25 m/min, and the mice again performed three sets. Thus, the treadmill speed continued to rise by 5 m/min until exhaustion. Exhaustion was defined as the point at which mice stayed on the grid at the back of the treadmill for a period of 30 s despite being given mild touches.

Microdialysis

A stainless steel guide cannula (EICOM CORP, Japan) was implanted stereotaxically into mice dorsal hippocampus (−1.8 mM anteroposterior, ±1.8 mM mediolateral, −1.9 mM dorsoventral from the bregma) following a previously reported method (Ishikawa et al., 2016). The mice were allowed to recover for 5 days after surgery. After that, the microdialysis probe (EICOM, CX-4-01) was inserted into the guide cannula approximately 120 min before the experiment. The probe was perfused with Ringer's solution, containing: 147 mM NaCl, 4 mM KCl, 2.3 mM CaCl2; pH 6.5.

The microdialysis probe was connected to a commercially available microdialysis liquid-swivel (EICOM) to ensure the free movement of the mice. For measuring hippocampal extracellular lactate concentration, ICR male mice ($n = 4$) were injected with sodium lactate dissolved in PBS (pH 7.4) at a dose of 2 g/kg BW by I.P. injection as previously described (Lezi et al., 2013). Another series of ICR male ($n = 5$) mice received the UK5099 (0.1 ml; 50 μmol/kg BW; Tocris, United Kingdom) 30 min before lactate injection. The flow rate was 1 μl/min allowing the collection of 10 μl samples every 10 min. Microdialysis samples were collected into ice-cooled polyethylene tubes (EICOM) using an EFC-96 fraction collector (EICOM). All other microdialysis samples were frozen immediately and stored at −80°C until lactate analysis.

Time Course of Changes in Blood Lactate Concentration After I.P. Injection

ICR male mice ($n = 6$) were injected with sodium lactate dissolved in PBS (pH 7.4) at a dose of 2 g/kg BW by I.P. injection. Lactate levels in blood obtained from the tail vein were measured using a portable blood lactate analyzer (Lactate Pro 2, Arkray, Tokyo, Japan) at pre-injection (Pre), 0, 5, 10, 15, 30, 60, and 180 min after sodium lactate injection.

Experimental Design

Experiment 1

The effects of a single bout of exercise at low, moderate, or high intensity on blood lactate, hippocampal PGC-1α mRNA, and mtDNA levels are summarized in **Figure 1**.

ICR male mice were divided into four groups: non-exercise (Non-ex, $n = 9$), low-intensity exercise (Low, $n = 8$–10/group), moderate-intensity exercise (Moderate, $n = 8$–10/group), and high-intensity exercise (High, $n = 8$–10/group). Mice blood lactate levels were measured before and immediately after every single bout of exercise. Mice were killed 12, 24, 48, and 72 h after each bout of exercise, and the hippocampus was quickly excised, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

Experiment 2

The effects of UK5099 administration on blood lactate, hippocampal mitochondrial biogenesis, MCTs, and BDNF mRNA levels after lactate administration or exercise are summarized in **Figures 2**–**4**.

ICR male mice were divided into three groups: control (Cont), vehicle + lactate (Lac), and UK5099 + lactate (UK5099 + Lac). UK5099 (0.1 ml; 50 μmol/kg BW; Tocris,

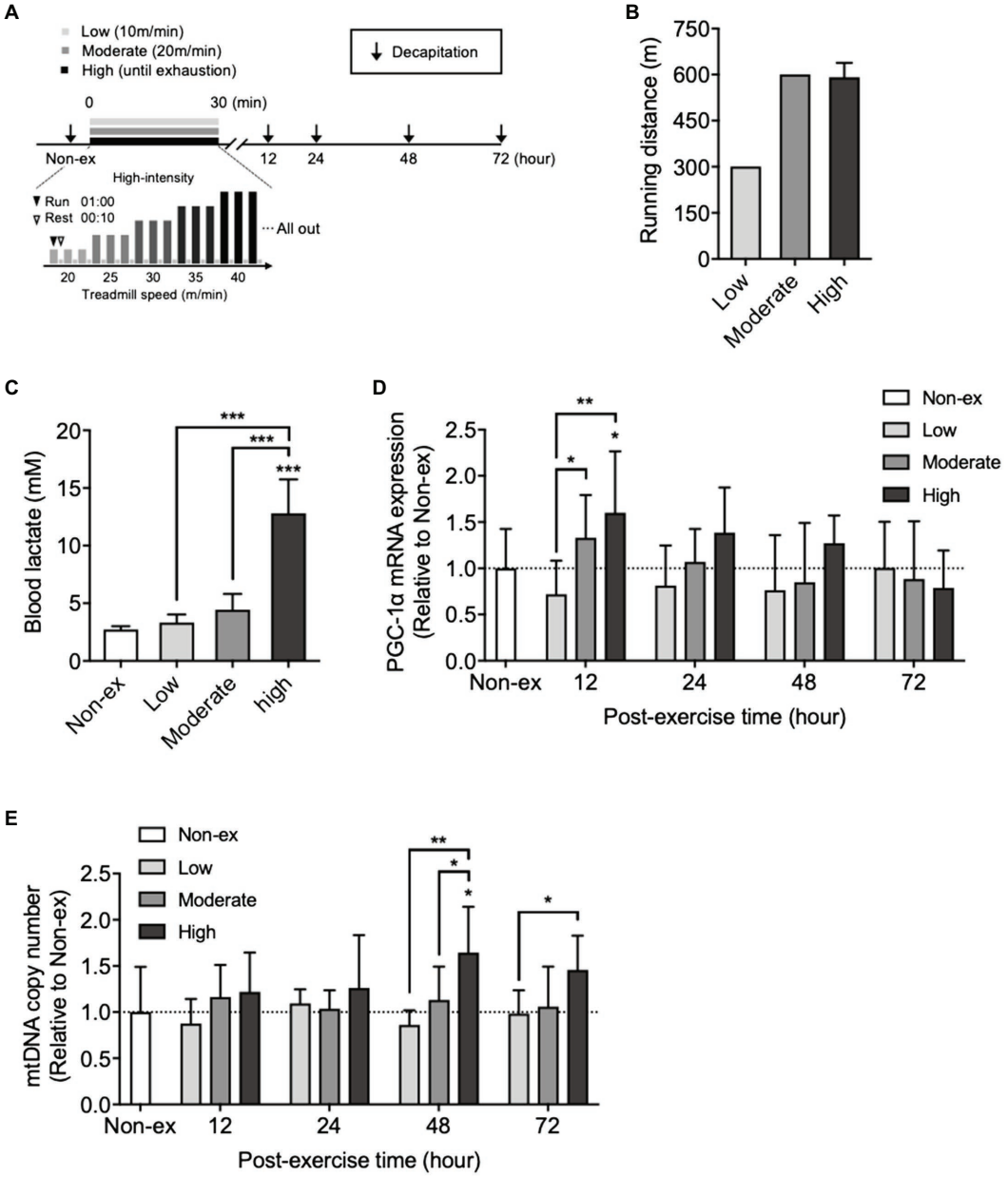

FIGURE 1 | Effects of a single bout of exercise performed at three different intensities on peroxisome proliferator-activated receptor gamma-coactivator 1α (PGC-1α) mRNA expression and mitochondrial DNA (mtDNA) copy number in the hippocampus and on blood lactate levels. **(A)** Protocols for a single bout of exercise. The mice ran on a treadmill at three different intensities: low intensity (low): 10 m/min for 30 min; moderate intensity: 20 m/min for 30 min (moderate); and high intensity: the treadmill speed was initially set at 20 m/min and gradually increased by 5 m/min until the mice were exhausted. **(B)** Running distance for 30 min at low- and moderate-intensity exercise groups and exhaustion at high-intensity exercise group. **(C)** Blood lactate levels immediately after a single bout of exercise at three different exercise intensities. **(D)** Time course changes in PGC-1α mRNA expression, and **(E)** mtDNA copy number in the hippocampus at non-exercise (Non-ex), 12, 24, 48, and 72 h after three different exercise intensities (Non-ex, $n = 9$; low, $n = 8$–10/group; moderate, 8–10/group; high, 8–10/group). All data were presented as the mean ± SD values. Data were analyzed using one-way ANOVA with Tukey's *post-hoc* tests or two-way ANOVA with Bonferroni's *post-hoc* tests. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ in comparison with the non-exercise group if not otherwise indicated.

United Kingdom), or a similar amount of DMSO, was I.P. injected 30 min before lactate injection (2 g/kg). Another series of ICR male mice were divided into three groups: Cont, vehicle + a single bout of high-intensity exercise (Ex), and UK5099 + a single bout of high-intensity exercise (UK5099 + Ex). Lactate levels in blood were measured before lactate administration and exercise and then 15 min after lactate administration or immediately after exercise. Mice were killed for the measurement of PGC-1α mRNA expression 12 h after lactate administration (Cont, $n = 9$; Lac, $n = 9$; UK5099 + Lac, $n = 8$) and exercise (Cont,

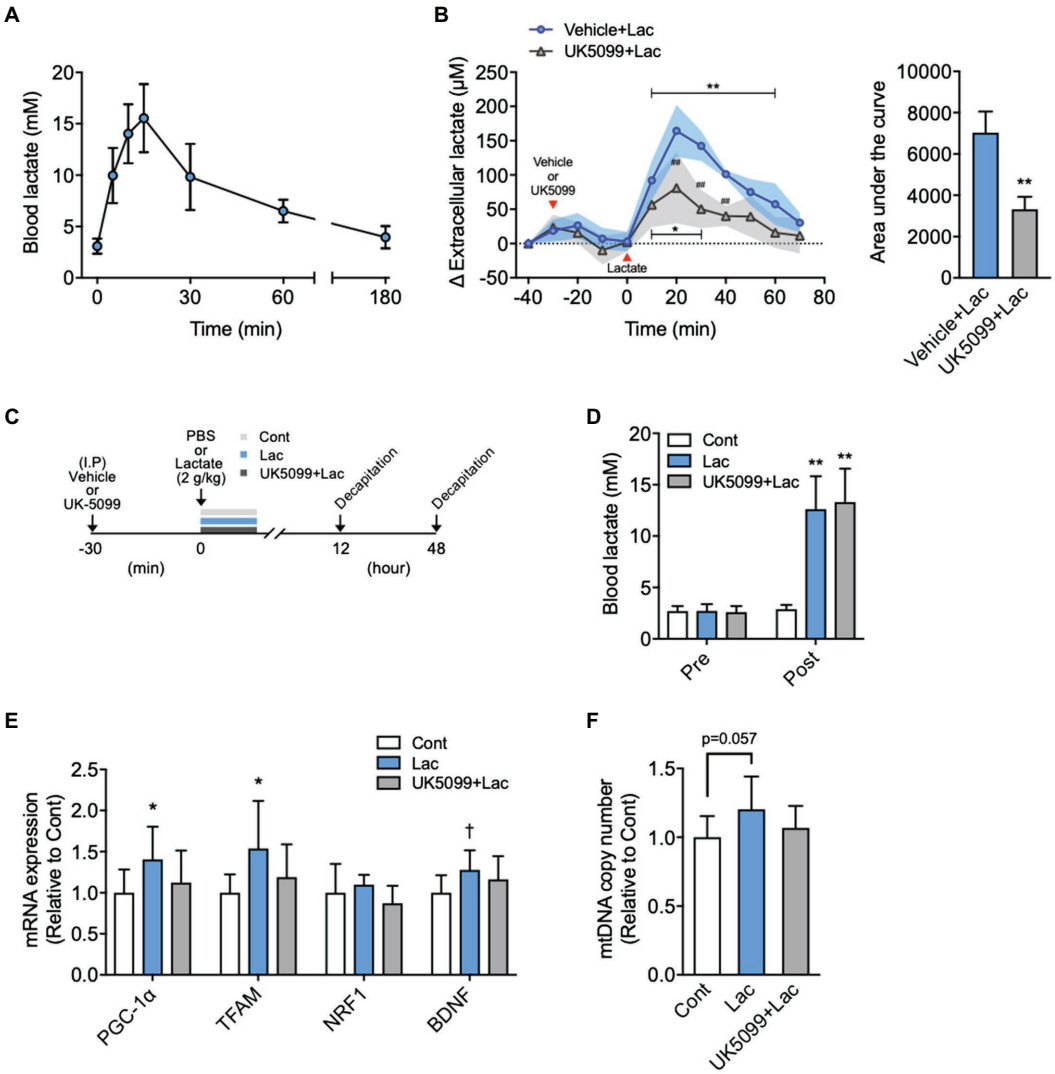

FIGURE 2 | Effects of lactate administration on hippocampal extracellular lactate concentration and mitochondrial biogenesis in the hippocampus. **(A)** Time course changes in blood lactate concentration after I.P. lactate injection (2 g/kg; $n = 6$). **(B)** The hippocampal extracellular lactate concentration changes after I.P. lactate injection with saline or monocarboxylate transporters (MCT) inhibitor (UK5099) administration and area under the curve (Vehicle + Lac, $n = 4$; UK5099 + Lac, $n = 5$). **(C)** Experimental design: Mice were injected saline or UK5099 by I.P. injection 30 min before lactate administration. Blood lactate concentration was measured 15 min after I.P. lactate or saline injection. **(D)** Blood lactate concentrations before I.P. lactate injection (Pre) and after lactate injection with and without UK5099 (Post). **(E)** PGC-1α, mitochondrial transcription factor A (TFAM), nuclear respiratory factor1 (NRF1), and brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus 12 h after I.P. lactate injection with and without UK5099 (Cont, $n = 9$; Lac, $n = 9$; UK5099 + Lac, $n = 8$). **(F)** mtDNA copy number in the hippocampus 48 h after I.P. injection of lactate with and without UK5099 (Cont, $n = 10$; Lac, $n = 10$; UK5099 + Lac, $n = 8$). All data were presented as the mean ± SD values. Data were analyzed using two-way ANOVA with Bonferroni's *post-hoc* tests and unpaired *t*-test (AUC) **(B)** and one-way ANOVA with Tukey's *post-hoc* tests **(D–F)**. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{\dagger}p < 0.1$ in comparison with baseline or Cont group and $^{\#\#}p < 0.01$ compared to Vehicle + Lac group.

$n = 9$; Ex, $n = 10$; UK5099 + Ex, $n = 8$) and for the mtDNA copy number 48 h after that administration (Cont, $n = 10$; Lac, $n = 10$; UK5099 + Lac, $n = 8$) and exercise (Cont, $n = 10$; Ex, $n = 10$; UK5099 + Ex, $n = 10$). The hippocampus was quickly excised, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

Surgery for Intra-Hippocampus Injection

After the treadmill habituation period, the mice were anesthetized with isoflurane and positioned in a stereotaxic apparatus for steel cannula placement (Narishige Co., Japan). For intra-hippocampal injection, a small hole was made using a dental drill, and a steel cannula (22-gauge) was inserted into the dorsal hippocampus. The guide cannula was located in the hippocampus (−1.8 mM anteroposterior, ±1.8 mM mediolateral, and −1.9 mM dorsoventral from the bregma) following a previously reported method (Ishikawa et al., 2016) and was fixed to the skull using anchor screws (EICOM) and dental cement. After surgery, the mice were individually housed in a warm cage and allowed to recover completely for at least 3 days.

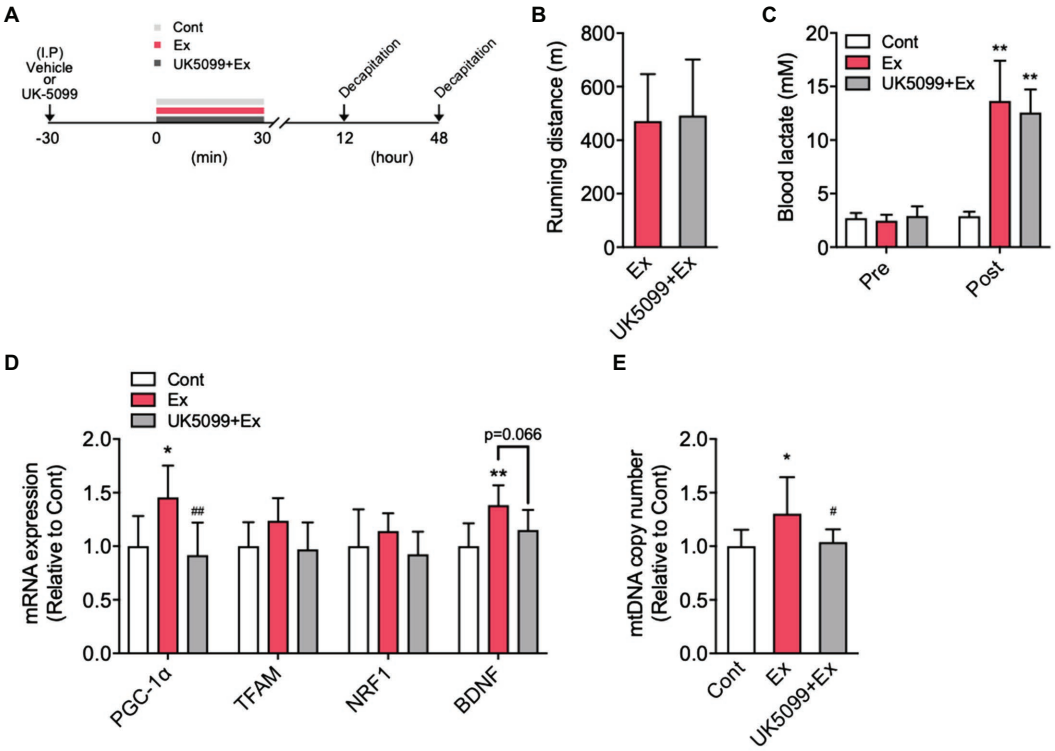

FIGURE 3 | Exercise-induced lactate release increased PGC-1α mRNA expression and mtDNA copy number in the hippocampus. **(A)** Experimental design: Mice were injected with saline or UK5099 by I.P. injection 30 min before a single bout of high-intensity exercise. Blood lactate concentrations were measured immediately after exercise. **(B)** Running distance to fatigue on a treadmill for mice administered saline (Ex) or UK5099 (UK5099 + Ex) before exercise. **(C)** Blood lactate concentrations before exercise (Pre) and immediately after exercise (Post). **(D)** PGC-1α, TFAM, NRF1, and BDNF mRNA expression in the hippocampus 12 h after exercise (Cont, $n = 9$; Ex, $n = 10$; UK5099 + Ex, $n = 8$). **(E)** mtDNA copy number in the hippocampus 48 h after exercise (Cont, $n = 10$; Ex, $n = 10$; UK5099 + Ex, $n = 10$). All data were presented as the mean ± SD values. Data were analyzed using one-way ANOVA with Tukey's *post-hoc* tests. $^{*}p < 0.05$ and $^{**}p < 0.01$ in comparison with Cont group and $^{\#\#}p < 0.05$ and $^{\#\#}p < 0.01$ in comparison with Ex group.

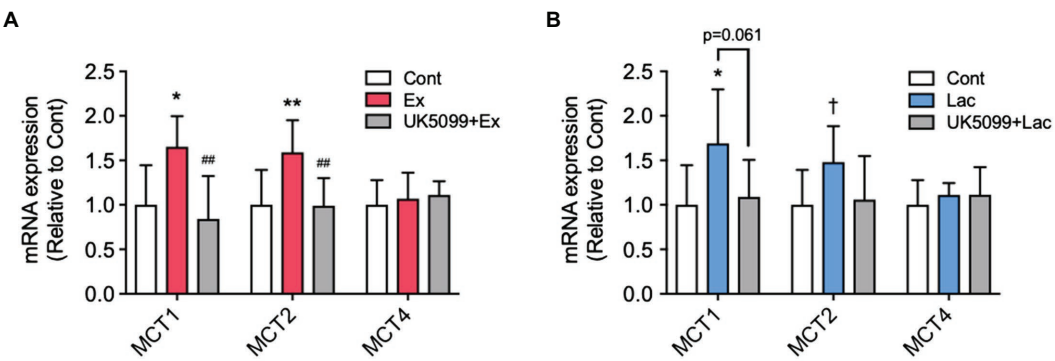

FIGURE 4 | The effects of UK5099 on the hippocampal MCTs and BDNF mRNA expression after lactate administration and high-intensity exercise. Mice were injected with saline or UK5099 by I.P. injection 30 min before a single bout of high-intensity exercise and lactate administration. MCT1, MCT2, and MCT4 mRNA expression in the hippocampus 12 h after **(A)** exercise (Cont, $n = 10$; Ex, $n = 9$; UK5099 + Ex, $n = 9$) and **(B)** I.P. injection of lactate (Cont, $n = 9$; Lac, $n = 9$; UK5099 + Lac, $n = 8$). All data were presented as the mean ± SD values. Data were analyzed using one-way ANOVA with Tukey's *post-hoc* tests. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{\dagger}p < 0.1$ in comparison with Cont group and $^{\#\#}p < 0.01$ in comparison with Ex group.

Experiment 3

The effects of pre-exercise intra-hippocampal injection of DAB on hippocampal glycogen and lactate concentrations and hippocampal PGC-1α and MCTs mRNA expression are summarized in **Figure 5**.

After surgery for intra-hippocampal injection, ICR male mice were divided into three groups: control (Cont, $n = 6$), saline + a single bout of high-intensity exercise group (Ex, $n = 6$), and DAB + a single bout of high-intensity exercise group (DAB + Ex, $n = 6$).

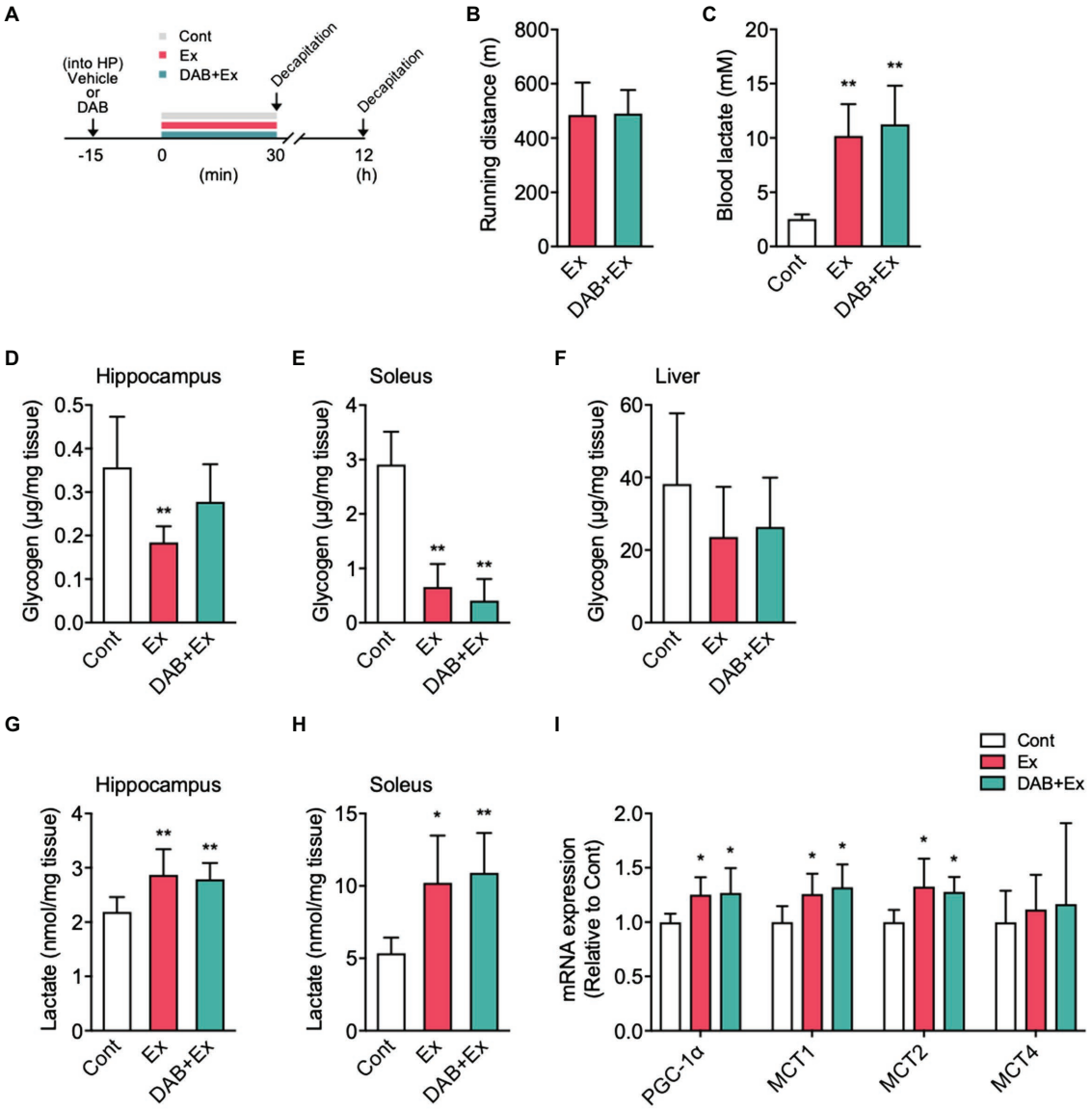

FIGURE 5 | The effects of intra-hippocampal 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) injection before high-intensity exercise on hippocampal glycogen, lactate levels, and PGC-1α, MCTs mRNA expression. **(A)** Experimental design: Mice were injected saline or DAB by intra-hippocampal injection 15 min before a single bout high-intensity exercise by using a micro-injection pump. **(B)** Running distance to fatigue on a treadmill for mice administered vehicle (Ex) or DAB (DAB + Ex) before exercise. **(C)** Blood lactate concentrations immediately after exercise and control. **(D)** Glycogen levels in the hippocampus, **(E)** soleus muscle **(F)** liver, **(G)** lactate levels in the hippocampus, and **(H)** soleus muscle immediately after exercise, with vehicle (Ex) or DAB (DAB + Ex) and control (Cont, $n = 6$; Ex, $n = 6$; DAB + Ex, $n = 6$). **(I)** PGC-1α, MCTs mRNA expression in the hippocampus 12 h after a single bout of high-intensity exercise (Cont, $n = 6$; Ex, $n = 7$; DAB + Ex, $n = 7$). All data were presented as the mean ± SD values. Data were analyzed using one-way ANOVA with Tukey's *post-hoc* tests. $^*p < 0.05$ and $^{**}p < 0.01$ compared to Cont group.

DAB (0.25 M in 1 μl of 0.9% saline; Wako, Osaka, Japan) or 1 μl of saline was injected into the hippocampus using a syringe pump (Legato 101, KD Scientific). Fifteen minutes after the injection, mice were subjected to a single bout of high-intensity exercise. Mice were killed by decapitation, and the hippocampus, soleus, and liver were excised immediately after exercise, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Another series of ICR male mice were divided into Cont ($n = 6$), Ex ($n = 6$), and DAB + Ex ($n = 6$). After intra-hippocampal injection of saline or DAB, mice were subjected to exercise. Mice were killed by decapitation, and the hippocampus was collected 12 h after high-intensity exercise, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

The hippocampus samples of mice were homogenized in TRIzol Reagent (Invitrogen, CA, United States) on ice, and total RNA

was extracted according to the manufacturer's instructions. Total RNA was quantified using absorption at 260 nm and the 260:280 nm ratio to assess concentration and purity. Complementary DNA was synthesized using 1 μg of total RNA in a 20 μl reaction with the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (FSQ-301; Toyobo, Osaka, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR was performed with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX Connect Real-time PCR system (Bio-Rad) to quantify the mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The mouse-specific primers used were as follows: PGC-1α: forward 5′-ACCCTGCCATTGTTAAGACC-3′, reverse 5′-CTGCTGCT GTTCCTGTTTTC-3′; TFAM: forward 5′-GAAGGGAATGG GAAAGGTAGA-3′, reverse 5′-AACAGGACATGGAAAGCA GAT-3′; NRF1: forward 5′- ATCCGAAAGAGACAGCAGACA-3′, reverse 5′- TGGAGGGTGAGATGCAGAGTA-3′; MCT1: forward 5′-TTGTCTGTCTGGTTGCGGCTTGATCG-3′, reverse 5′-GCC CAAGACCTCCAATAACACCAATGC-3′; MCT2: forward 5′-CACCACCTCCAGTCAGATCG-3′, reverse 5′-CTCCCACTA TCACCACAGGC-3′; MCT4: forward 5′-TCAATCATGGTGCT GGGACT-3′, reverse 5′-TGTCAGGTCAGTGAAGCCAT-3′; BDNF: forward 5′-TGCAGGGGCATAGACAAAAGG-3′, reverse 5′-CTTATGAATCGCCAGCCAATTCTC-3′; and GAPDH forward 5′-CATCACTGCCACCCAGAAGA-3′, reverse 5′-ATG TTCTGGGCAGCC-3′. The $2^{-\Delta\Delta C}{}_t$ method was used to analyze relative mRNA expression values (Livak and Schmittgen, 2001). Sample analysis for each gene was performed in duplicate.

mtDNA Copy Number

For quantifying hippocampal mtDNA, total DNA was extracted from the hippocampus using the phenol-chloroform method, as described previously (Mikami et al., 2021). First, the hippocampal tissue (~20 mg) was dissolved in 200 μl of lysate buffer containing 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA (pH 8.0), 0.1% SDS, and 2.5% proteinase K; next, it was vortexed, incubated at 56°C for 90 min, and centrifuged at 2,000 *g* for 10 min.

The supernatant was mixed with an equal volume of phenol/chloroform/isoamyl alcohol, vortexed, and centrifuged at 2,000 × *g* for 10 min. Then, the supernatant obtained was mixed with two volumes of 100% ethanol by slow inversion and centrifuged at 10,000 × *g* for 10 min. After the supernatant was removed, the precipitate was dissolved in 70% ethanol by slow inversion and centrifuged at 10,000 × *g* for 10 min.

Subsequently, after the supernatant obtained was removed, the precipitate was left for 5 min at room temperature, dissolved in Tris-EDTA (TE) buffer (pH 8.0), and stored at −20°C until analysis. DNA concentration and purity were analyzed using absorption at 260 nm and the 260:280 nm absorption ratio, respectively.

Quantitative real-time PCR (10 ng DNA) was performed with the SsoAdvanced Universal SYBR Green Supermix. 18S ribosomal RNA (18S rRNA) was used as the nuclear DNA (nDNA) control. The mtDNA and nDNA primers used were as follows: COX I: forward 5′-TGATTCCCATTATTTT CAGGCTTC-3′, reverse 5′-ACTCCTACGAATATGATGGCG AA-3′; and 18S rRNA: forward 5′-CGCCGCTAGAG GTGAAATTC-3′, reverse 5′-CTTGGCAAATGCTTTCGCTC-3′. The $2^{-\Delta\Delta C}{}_t$ method was used to analyze the relative mtDNA to nDNA copy number ratio (Livak and Schmittgen, 2001). Sample analysis for each gene was performed in duplicate.

Lactate and Glycogen Concentrations in Tissues

For measuring lactate and glycogen concentrations, the mice were killed immediately after a single bout of high-intensity exercise, and the hippocampus, muscle, and liver tissues were obtained. Glycogen and lactate levels in these tissues were colorimetrically measured using Glycogen Assay Kit II (ab169558; Abcam, Cambridge, United Kingdom) and L-Lactate Assay Kit (MAK064; Sigma, United Kingdom), respectively, according to the manufacturer's protocols. Samples were deproteinized with a 10 kDa MWCO spin filter to remove lactate dehydrogenase.

Statistical Analysis

Data are expressed as mean ± SD and were analyzed using GraphPad Prism version 8.4.1 (MDF Co, Ltd., Tokyo, Japan). Group comparisons were performed using one-way ANOVA with Tukey's *post-hoc* tests or two-way ANOVA with Dunnett's and Bonferroni's *post-hoc* tests. In addition, comparisons of two groups were performed using an unpaired Student's *t*-test. The differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Time Course of Changes in Hippocampal PGC-1α mRNA Levels and mtDNA Copy Number Following a Single Bout of Exercise

We examined the time course changes in hippocampal PGC-1α mRNA and mtDNA copy number after a single bout of exercise at three different intensities (**Figure 1A**). Low- and moderate-intensity exercise groups ran 300- and 600-meter distances in 30 min, respectively. Although running distance until exhaustion in the high-intensity exercise group (time to exhaustion: 26.1 ± 0.37 min, including rest) did not differ from the distance covered by the moderate-intensity exercise group (**Figure 1B**), the blood lactate concentrations immediately after high-intensity exercise were significantly higher than those in the other two groups ($F_{3,36} = 80.35$, $p < 0.0001$; **Figure 1C**). Hippocampal PGC-1α mRNA levels did not significantly change at any time point after the low-intensity exercise. At 12 h after moderate- and high-intensity exercises, the expression levels were significantly higher than those of the low-intensity exercise group. Compared with the non-exercise group, PGC-1α mRNA levels significantly increased only in the high-intensity exercise group ($F_{2,127} = 5.26$, $p = 0.0065$; **Figure 1D**). The mtDNA copy number significantly

increased 48 h after a single bout of high-intensity exercise compared with the non-exercise, low-, and moderate-intensity exercise groups ($F_{2,129} = 10.70$, $p < 0.0001$). However, the low- or moderate-intensity exercise groups did not show any increase compared to the non-exercise group (**Figure 1E**). These results indicated a relationship between the increase in blood lactate and hippocampal PGC-1α mRNA levels observed after high-intensity exercise.

The Effects of a Single Administration of Lactate on Hippocampal Extracellular Lactate Concentrations and Mitochondrial Biogenesis

Blood lactate levels reached 15.5 ± 1.36 mM at 15 min following I.P. lactate injection, comparable to that observed immediately after high-intensity exercise (**Figure 2A**), and returned to baseline levels at 180 min after injection. Baseline hippocampal extracellular lactate concentrations did not differ between the two groups (Vehicle + Lac, 99.1 ± 3.71; UK5099 + Lac, 107.3 ± 2.44 μm; $p = 0.11$). The extracellular lactate concentration in the hippocampus was significantly increased following I.P. lactate administration after 10 min and remained high until 60 min, and then returned to baseline.

At 20 min after lactate injection, hippocampal extracellular lactate concentration increased by ~160 μm from baseline. Lactate-derived elevation in extracellular lactate concentration was significantly suppressed by UK5099 injection ($F_{1,82} = 44.00$, $p < 0.0001$; **Figure 2B**). The decrease in hippocampal extracellular lactate *via* UK5099 administration was also proved by the results of the area under the curve analysis (**Figure 2B**). We administered vehicle or UK5099 to the mice 30 min before lactate injection (**Figure 2C**). We observed no difference in the increased blood lactate levels between Lac and Lac + UK5099 group, which were equal to those observed immediately after high-intensity exercise (**Figure 2D**). The mice injected with lactate showed significantly higher PGC-1α ($F_{2,23} = 3.33$, $p = 0.051$) and TFAM ($F_{2,23} = 3.27$, $p = 0.058$) mRNA, but not significantly higher in BDNF mRNA ($F_{2,23} = 2.61$, $p = 0.098$). Besides, these mice tended to have a higher mtDNA copy number ($F_{2,25} = 2.98$, $p = 0.068$) in the hippocampus than the Cont group after injection. However, these changes were not observed in mice injected UK5099 (**Figures 2E**,**F**).

The Effects of UK5099 Administration Before Exercise on Hippocampal Mitochondrial Biogenesis

Mice were injected vehicle or UK5099 before a single bout of high-intensity exercise (**Figure 3A**). Ex and UK5099 + Ex group showed a similar running distance to fatigue (**Figure 3B**) and blood lactate levels immediately after exercise (**Figure 3C**). However, the Ex group showed significantly higher hippocampal PGC-1α ($F_{2,24} = 9.67$, $p < 0.001$) and BDNF mRNA (12 h after exercise; $F_{2,24} = 7.89$, $p < 0.01$), and hippocampal mtDNA copy number (48 h after exercise; $F_{2,27} = 5.24$, $p < 0.05$) than the Cont group (**Figures 3D**,**E**). In contrast, the UK5099 + Ex group showed significantly lower PGC-1α mRNA and mtDNA levels than the Ex group (**Figures 3D**,**E**). However, there was no significant difference in downstream regulators of mitochondrial biogenesis, TFAM, and NRF1 mRNA among the groups (**Figure 3D**).

The Effects of UK5099 on Hippocampal MCTs mRNA Expression After Lactate Administration and Exercise

We found that MCT1 ($F_{2,24} = 8.12$, $p < 0.01$) and MCT2 ($F_{2,24} = 7.43$, $p < 0.01$) mRNA expression in the hippocampus significantly increased in the Ex group than in the Cont group, whereas these increments were abolished by UK5099 administration before exercise (**Figure 4A**). After lactate administration, only MCT1 ($F_{2,23} = 4.59$, $p < 0.05$) increased significantly, and MCT2 ($F_{2,23} = 2.94$, $p = 0.075$) tended to be higher than that in the Cont group; however, the increment of those genes was not observed in mice injected UK5099 compared with the Cont group (**Figure 4B**). On the other hand, no changes were observed in MCT4 mRNA expression after exercise and lactate administration (**Figures 4A**,**B**).

The Effects of Glycogenolysis Inhibition During Exercise on Hippocampal Lactate and the Expression of PGC-1α and MCTs mRNA

Mice received a micro-injection of DAB or vehicle into the hippocampus, and 15 min later, mice were subjected to a single bout of high-intensity exercise (**Figure 5A**). Mice injected with vehicle or DAB showed a similar running distance to fatigue (**Figure 4B**). Immediately after exercise, the blood lactate levels were similar with and without DAB injection ($F_{2,17} = 19.02$, $p < 0.0001$; **Figure 5C**). The hippocampal glycogen levels significantly decreased in the Ex group compared with the Cont mice. Still, significant alterations were not noted in the DAB + Ex group, indicating the pre-exercise micro-injection of DAB inhibited hippocampal glycogenolysis during exercise ($F_{2,15} = 6.03$, $p < 0.05$; **Figure 5D**). The glycogen concentration in mice soleus muscles from the Ex and DAB + Ex groups were decreased compared with Cont group ($F_{2,15} = 48.01$, $p < 0.0001$; **Figure 5E**). However, liver glycogen concentration did not significantly decrease in either group during a single short-term, high-intensity exercise for approximately 30 min (**Figure 5F**).

Unexpectedly, despite the inhibition of hippocampal glycogenolysis during exercise by DAB, hippocampal lactate levels immediately after exercise showed a similar significant increase in both Ex and DAB + Ex groups. These results indicated that the increase in hippocampal lactate during high-intensity exercise could much depend on lactate uptake from the circulating blood than intra-hippocampal lactate production by astrocyte glycolysis ($F_{2,15} = 6.57$, $p < 0.01$; **Figure 5G**). Additionally, in the soleus muscle, lactate levels noted immediately after a single bout of high-intensity exercise were significantly higher in both exercise groups than in the Cont group ($F_{2,15} = 8.52$,

$p < 0.01$; **Figure 5H**). Furthermore, the exercise groups with or without DAB injection showed a similar higher PGC-1α ($F_{2,17} = 5.29$, $p < 0.05$), MCT1 ($F_{2,17} = 5.58$, $p < 0.05$), and MCT2 mRNA expression ($F_{2,17} = 5.88$, $p < 0.05$) in the hippocampus than the Cont group, but MCT4 mRNA expression did not differ among the three groups (**Figure 5I**). These results suggested that the single short-term, high-intensity exercise used in this study rapidly increases lactate release from skeletal muscle into blood, which is pivotal to increase hippocampal lactate concentration and might contribute to enhance the induction of PGC-1α and MCTs mRNA expression in the hippocampus.

DISCUSSION

Regular exercise training enhances skeletal muscle and hippocampal PGC-1α mRNA and mtDNA copy number (Zhang et al., 2012; Halling et al., 2019). Six weeks of HIIT accompanied by increased blood and hippocampal lactate concentrations has been reported to increase hippocampal expression of mitochondrial biogenesis-related genes PGC-1α, NRF2, and mtDNA copy number (Hu et al., 2021). The effects of regular exercise reflect an accumulation of physiological adaptations produced by bouts of high-intensity exercise. Thus, it is essential to understand the beneficial effects of a single bout of high-intensity exercise and elucidate the mechanisms underlying their effects to produce better high-intensity exercise training strategies. However, the effects of acute high-intensity exercise on hippocampal mitochondrial biogenesis are unclear.

In the current study, an increase in hippocampal PGC-1α mRNA expression was observed 12 h after exercise. An increase in hippocampal mtDNA copy number followed 48 h later, and its increment depended on exercise intensity. Running time to exhaustion (26.1 ± 0.37 min) is slightly below in the high-intensity exercise group compared to the low- and moderate-intensity exercise groups (30 min); running distance in the high-intensity group did not differ from that of the moderate-intensity exercise group. However, the blood lactate concentrations immediately after exercise and post-exercise hippocampal mitochondrial biogenesis significantly differed between the three exercise groups. These results are consistent with the previous reports that 20 min of a single bout of high-intensity exercise induced the activation of skeletal muscle mitochondrial transcription (Hoshino et al., 2015), and exercise intensity affects skeletal muscle PGC-1α protein levels (Terada et al., 2005; Tadaishi et al., 2011). Moreover, the present results are consistent with the results of the human study, which indicated that a high-intensity interval cycle ergometer for a total duration of 20 min showed higher BDNF serum concentration levels than at rest and moderate-intensity exercise group in the human study (Saucedo Marquez et al., 2015).

A blood lactate concentration >12 mM was required to increase hippocampal PGC-1α mRNA and mtDNA copy number. In contrast, low- or moderate-intensity exercise correlated with 3–4 mM blood lactate concentration, which did not increase the hippocampal PGC-1α mRNA and mtDNA copy number. These results are consistent with previous findings that exercise intensity above the LT increased PGC-1α mRNA expression in human skeletal muscle but that below the LT did not (Tobina et al., 2011). Thus, these results suggested that elevated blood lactate induced by high-intensity exercise above the LT would contribute to the induction of mtDNA copy number through PGC-1α expression in the hippocampus.

To our knowledge, the current study is the first to show that an increase in mitochondrial biogenesis in the brain, especially the hippocampus, depends on exercise intensity in the same manner as seen in skeletal muscle. In contrast, a single bout of low- or moderate-intensity exercise did not affect mitochondrial biogenesis in the hippocampus. Thus, single bouts of low- or moderate-intensity exercise are not likely to induce hippocampal mitochondrial biogenesis, suggesting that these exercises would need to be regularly repeated over 4 weeks to promote hippocampal mitochondrial biogenesis (Steiner et al., 2011; Zhang et al., 2012).

Brain cell MCTs transport lactate across the BBB (El Hayek et al., 2019), which is enhanced during exercise (Quistorff et al., 2008; van Hall et al., 2009). Acute (Takimoto and Hamada, 2014) and chronic exercise (El Hayek et al., 2019) above LT enhances MCT expression in the hippocampus, enhancing cognitive function by increasing BDNF expression through the induction of PGC-1α (El Hayek et al., 2019). Besides, Overgaard et al. (2012) indicated that the brain uptakes lactate from the blood and releases lactate into the blood concomitantly, and that net lactate uptake is a plus, and net brain lactate uptake is increased by the increase in circulating blood lactate level during high-intensity exercise. Based on these data, we predicted that some of the blood lactate increased by a single bout of high-intensity exercise or lactate administration would be taken up into the brain *via* MCTs, where it would promote PGC-1α expression, followed by mitochondrial biogenesis. To test this hypothesis, we examined hippocampal extracellular lactate concentration by using *in vivo* microdialysis in the presence and absence of UK5099, a potent MCT1–4 inhibitor, after lactate injection. In this experiment, to eliminate the influence of lactate production derived from astrocytes glycogenolysis, which would be expected to occur during high-intensity exercise, I.P. lactate administration and microdialysis were performed using the resting instead of the exercised mice.

We found that the hippocampal extracellular lactate concentration increased following lactate injection, consistent with previous studies (Machler et al., 2016; Carrard et al., 2018). However, this lactate concentration increase was suppressed by UK5099 administration, indicating that MCT inhibition prevents lactate transport from the blood into hippocampal extracellular space. Furthermore, UK5099 administration also suppressed lactate-induced increases in hippocampal PGC-1α and TFAM mRNA, and mtDNA copy number.

The results demonstrate that high-intensity exercise or lactate administration increases hippocampal extracellular lactate concentration, which could induce hippocampal PGC-1α mRNA expression and increase mtDNA copy number. Moreover, hippocampal BDNF mRNA expression was also increased after a single bout of high-intensity exercise, which was consistent with a previous study showing that increased PGC-1α led to

the induction of BDNF expression (Wrann et al., 2013; Mikami et al., 2021). In addition, the lactate-induced hippocampal BDNF expression was suppressed by injecting MCT1/2 inhibitor, AR-C155858 (El Hayek et al., 2019). However, the present study does not elucidate how the increased hippocampal lactate upregulated PGC-1α and BDNF mRNA expression. Yang et al. showed that exposure of neurons to lactate modified the intracellular NADH/NAD ratio, promoted N-methyl-D-aspartate (NMDA) receptor activity and its downstream signaling cascade Erk1/2 (Yang et al., 2014). The activating of the NMDA receptors caused an increase in intracellular Ca^{2+} concentration, thereby inducing the gene expression of PGC-1α (Luo et al., 2009). We hypothesize that lactate taken up from blood into the brain could have altered intracellular NADH/NAD ratio and could have triggered changed mRNA expressions related to mitochondrial biogenesis. Further studies are necessary to prove our hypothesis.

Exogenous lactate administration increased blood lactate concentration, induced hippocampal PGC-1α and MCT mRNA expression, and increased mtDNA copy number, similar to high-intensity exercise. These results indicated that systemic administration of lactate mimics the effect of high-intensity exercise on hippocampal mitochondria biogenesis. However, a single lactate administration was insufficient to increase MCT2 and BDNF mRNA, and mtDNA copy number to the same extent as a single bout of high-intensity exercise.

We speculate that the differences in hippocampal mRNA expression and mtDNA copy number between exogenous lactate administration and bouts of high-intensity exercise may result from the production of myokines and other factors induced by high-intensity exercise but not by exogenous lactate administration. Irisin, a myokine released from skeletal muscle, is transferred to the brain and induces hippocampal PGC-1α and BDNF expression (Wrann et al., 2013; Azimi et al., 2018; Lourenco et al., 2019). In the future studies, we plan to investigate the effect of lactate administration on myokines in the circulating blood.

The role of lactate produced by astrocytic glycolysis in mediating memory processes (Descalzi et al., 2019) and exercise endurance capacity has recently been characterized (Machler et al., 2016; Matsui et al., 2017). The glycogen in astrocytes is broken down and metabolized to lactate, shuttled to neurons *via* MCT2, and acts as an energy source in nerve cells (Pellerin et al., 1998; Brooks, 2020). Inhibiting hippocampal glycogenolysis *via* DAB injection impaired memory and decreased endurance exercise capacity, indicating that the lactate supplied by astrocytes to neurons is critical for regulating memory processing (Suzuki et al., 2011) and maintaining endurance capacity (Matsui et al., 2017). We speculate that glycogen-derived lactate released during exercise might mediate the promotion of hippocampal mitochondrial biogenesis after high-intensity exercise.

Our results showed that compared with mice injected with saline, DAB injection before exercise inhibited hippocampal glycogenolysis after exercise; however, hippocampal lactate concentration following high-intensity exercise was elevated in the presence or absence of DAB. Moreover, hippocampal PGC-1α, MCT1, and MCT2 mRNA expression following high-intensity exercise were unaffected by DAB injection before exercise. These results indicate that the lactate derived from hippocampal glycogenolysis during high-intensity exercise does not promote hippocampal PGC-1α, MCT1, and MCT2 mRNA expression. This was inconsistent with the previous findings that lactate derived from astrocytic glycogenolysis is vital for cognitive memory and regulating exercise performance. Based on these results, although a possible effect of brain glycogen-derived lactate release cannot be ruled out, we speculate that the induction of hippocampal PGC-1α, MCT1, and MCT2 mRNA expression after exhaustive and short-term exercise is attributable to the marked increase in the lactate taken into the brain from the blood, rather than from astrocytic glycogenolysis.

The following results support our speculation; (1) hippocampal extracellular lactate, taken up from blood into the brain, was suppressed by MCT inhibitors, followed by a marked decrease in PGC-1α, MCT1, and MCT2 mRNA expression; (2) the inhibition of hippocampal glycogenolysis by DAB did not affect PGC-1α, MCT1, and MCT2 mRNA expression. The type of exercise used in the present study was short-term and exhaustive; if the exercise were different, the contribution of lactate derived from brain glycogenolysis on gene expression related to mitochondrial biogenesis would be altered. Further study is necessary to investigate the contribution of lactate, which is incorporated from circulating blood or produced from brain glycogenolysis, and the beneficial effect of different types of exercises on brain function. Our results provide a helpful strategy to enhance brain function *via* high-intensity exercise training, which may contribute to developing exercise programs in human subjects.

Limitations

In the present study, we used only male mice, based on results from a previous study using male mice that showed that intermittent intense exercise prevents the stress-induced decline of cognitive function and suppresses the decrease in neurons survival (Lee et al., 2018). Thus, we did not compare the difference in hippocampal mitochondrial biogenesis after exercise between male and female mice. Therefore, the sex difference in mitochondrial biogenesis is not clarified. However, it has been reported that there are no differences in heart mitochondrial activity between sexes in young mice (6 weeks old). On the other hand, it has been reported that female mice have a higher brain mitochondrial respiratory and reserve function than male mice because of lower H_2O_2 production in female cardiac and brain tissue (Khalifa et al., 2017). Besides, it has been reported that, in old age mice (22 months old), the gene expression of key regulators of mitochondrial biogenesis (PGC-1α, Sirtuin1, and NRF2) and mitochondrial activity in the brain was significantly lower in females than males (Zawada et al., 2015). Until now, the sex differences in the effects of exercise on brain mitochondrial biogenesis have not been clarified. Therefore, further studies to examine whether mitochondrial biogenesis induced by high-intensity exercise is different between the sexes are needed.

This study focused only on lactate and did not consider stress-related factors, such as cortisol, (nor) epinephrine, and corticosterone. High-intensity exercise, such as the one used in the present study, increases the stress-related factors mentioned above. Chronic stress has previously been shown to have undesirable effects on brain functions. On the other hand, some studies have reported that acute stress, unlike chronic stress, has a beneficial effect on brain functions. For example, acute and short-term stress or single doses of corticosterone significantly increase hippocampal cell proliferation (Kirby et al., 2013). Additionally, acute stress increases the circulating corticosterone levels, leading to the activation of glucocorticoid receptors and subsequently provoking the synthesis and BDNF expression (Yang et al., 2004). Based on these results, stress-related factors might have also contributed to the increased mitochondrial biogenesis observed in this study. However, we did not examine the effects of high-intensity exercise-induced mitochondria biogenesis on stress-related factors such a corticosterone plasma concentration and PGC-1α alteration in the presence of corticosterone antagonist. Therefore, in addition to the effect of blood lactate on mitochondria biogenesis, the study of stress-related factors on increased mitochondria biogenesis after high-intensity exercise is necessary for the future.

CONCLUSION

Our results show that a single bout of exercise above the LT intensity enhanced hippocampal mitochondrial biogenesis. A single injection of exogenous lactate increased hippocampal extracellular lactate concentration, which partially mimicked the effects of a single bout of high-intensity exercise. Additionally, the UK5099 administration abolished the increase in hippocampal extracellular lactate concentration. However, DAB did not affect hippocampal lactate, PGC-1α, and MCT mRNA concentration despite inhibiting hippocampal glycogenolysis. Therefore, the lactate released into the blood from skeletal muscle during high-intensity exercise may be a crucial stimulator for hippocampal mitochondria biogenesis. These data improve our understanding of the effects of high-intensity exercise and may be a basis for further clinical or athletic applications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Nippon Medical School (approval no. 30-030).

AUTHOR CONTRIBUTIONS

JP and TM conceived and designed experiments, interpreted the results of experiments, and drafted the manuscript. JP, JK, and TM performed experiments and analyzed data. JP prepared figures. All authors have approved to submit the final version manuscript.

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REVIEW
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Lactate as an Astroglial Signal Augmenting Aerobic Glycolysis and Lipid Metabolism

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Astrocytes, heterogeneous neuroglial cells, contribute to metabolic homeostasis in the brain by providing energy substrates to neurons. In contrast to predominantly oxidative neurons, astrocytes are considered primarily as glycolytic cells. They take up glucose from the circulation and in the process of aerobic glycolysis (despite the normal oxygen levels) produce L-lactate, which is then released into the extracellular space *via* lactate transporters and possibly channels. Astroglial L-lactate can enter neurons, where it is used as a metabolic substrate, or exit the brain *via* the circulation. Recently, L-lactate has also been considered to be a signaling molecule in the brain, but the mechanisms of L-lactate signaling and how it contributes to the brain function remain to be fully elucidated. Here, we provide an overview of L-lactate signaling mechanisms in the brain and present novel insights into the mechanisms of L-lactate signaling *via* G-protein coupled receptors (GPCRs) with the focus on astrocytes. We discuss how increased extracellular L-lactate upregulates cAMP production in astrocytes, most likely *via* L-lactate-sensitive G_s-protein coupled GPCRs. This activates aerobic glycolysis, enhancing L-lactate production and accumulation of lipid droplets, suggesting that L-lactate augments its own production in astrocytes (i.e., metabolic excitability) to provide more L-lactate for neurons and that astrocytes in conditions of increased extracellular L-lactate switch to lipid metabolism.

Keywords: L-lactate, L-lactate sensitive receptors, astrocytes, cAMP, aerobic glycolysis, lipid metabolism

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INTRODUCTION

Historically, L-lactate was first considered as a cellular waste product of glycolytic metabolism, however, it was later proposed that L-lactate can also act as a supplemental oxidative energy substrate and as a signaling molecule in the brain (Dienel, 2012a; Magistretti and Allaman, 2018).

L-Lactate is involved in various cellular processes in the brain, including in the regulation of intracellular Ca^{2+} signaling (Requardt et al., 2012), cell energy metabolism (Bergersen and Gjedde, 2012; Barros, 2013), activity of various channels and transporters (Gordon et al., 2008; Ohbuchi et al., 2010), myelination (Fünfschilling et al., 2012), and gene expression (Yang et al., 2014; Descalzi et al., 2019). L-Lactate was shown to support high-level cognitive functions, learning and long-term memory formation (Newman et al., 2011; Suzuki et al., 2011; Gibbs, 2015; Murphy-Royal et al., 2020), and may have a neuroprotective role against excitotoxicity (Ros et al., 2001) and ischemia (Berthet et al., 2012; Castillo et al., 2015). Moreover, impaired

L-lactate signaling and metabolism have been associated with several brain pathologies, such as epilepsy (Yang et al., 2016), depression (Carrard et al., 2018), and neurodevelopmental disorders [e.g., X-linked intellectual disability (XLID); D'Adamo et al., 2021]. However, the molecular mechanisms of the broad spectrum of L-lactate functions in the brain in health and disease are not yet clear (Dienel, 2012a) and may involve the role of L-lactate as an energy substrate and as a signaling molecule in the brain.

The brain is composed of various cell types with distinct metabolic profiles. Under physiologic conditions, astrocytes and mature oligodendrocytes are considered mainly glycolytic cells, whereas neurons and microglia are predominantly oxidative (Fünfschilling et al., 2012; Zhang et al., 2014; Sharma et al., 2015; Afridi et al., 2020; Yang et al., 2021). A high glycolytic rate with L-lactate production is present in astrocytes and oligodendrocytes during increased neuronal activity despite normal brain oxygen levels, a process termed aerobic glycolysis (Pellerin and Magistretti, 1994; Barros, 2013; Saab et al., 2016), also known as the Warburg effect initially described in fast proliferating cancer cells (Warburg, 1956; Vander Heiden et al., 2009). To avoid L-lactate-mediated intracellular acidification, which causes negative feedback on glycolytic flux (Hertz et al., 2014), L-lactate is shuttled intra-/intercellularly and extracellularly by diffusion down its concentration gradient *via* membrane monocarboxylate transporters (MCTs; Pérez-Escuredo et al., 2016), K^+/voltage-sensitive cation channels (Sotelo-Hitschfeld et al., 2015), pannexin and connexin hemichannels (Karagiannis et al., 2016), and gap junctions (Scemes et al., 2017). Shuttling of brain L-lactate enables neurons to accept L-lactate and use it as an energy fuel in oxidative metabolism (Hertz et al., 2014). L-Lactate can also act as a signaling molecule intracellularly or extracellularly indirectly or *via* receptor-mediated signaling mechanisms (Barros, 2013; Mosienko et al., 2015). Due to its signaling characteristics and shuttling capability, L-lactate may act as a brain "volume transmitter", activating L-lactate-sensitive receptors (LLRs) on neural cells that are relatively distant from the site of L-lactate release. In this way, L-lactate-mediated signals could spread over larger areas of the brain (Bergersen and Gjedde, 2012).

In this review, we first discuss the current knowledge on intracellular and extracellular L-lactate signaling mechanisms in the brain with an emphasis on receptor-based signaling in astrocytes, where L-lactate is predominantly produced in the brain. Then, we discuss how extracellular L-lactate augments astroglial aerobic glycolysis and thus its own production and how this may contribute to L-lactate "volume transmission". The role of L-lactate as a metabolic substrate and signal in the control of brain lipid metabolism is also addressed.

MOLECULAR MECHANISMS OF L-LACTATE SIGNALING IN THE BRAIN

In the brain, L-lactate can exert its role as a signaling molecule through several intracellular and extracellular mechanisms.

Intracellular L-Lactate Signaling in the Brain

Once inside the cells, L-lactate can modulate brain function indirectly by changing the intracellular redox state of cells as glycolytic transformation of L-lactate into pyruvate generates NADH and thus increases the NADH/NAD^+ ratio (Hung et al., 2011; Hertz et al., 2014). By altering the cellular redox state, L-lactate (1) promotes the expression of synaptic plasticity-related genes, such as Arc, c-Fos, and Zif268, by potentiating ionotropic glutamate receptor (NMDA receptor)-mediated Ca^{2+} currents induced by glutamate and glycine leading to activation of a downstream Erk1/2 signaling cascade in neurons *in vitro* and *in vivo* (Yang et al., 2014) and (2) modulates astroglial Ca^{2+} signaling by increasing the frequency of dopamine-induced Ca^{2+} signals (Requardt et al., 2012). The entry of L-lactate into cells can also affect the cell energy status where L-lactate is first metabolized to pyruvate, which is then used for generation of ATP in the tricarboxylic acid cycle (TCA) leading to an increased ATP/ADP ratio. This was shown to regulate the activity of ATP-sensitive K^+ channels in hypothalamic and orexin neurons, which close when cytoplasmic ATP levels increase, leading to depolarization of the membrane (Song and Routh, 2005; Parsons and Hirasawa, 2010; Mosienko et al., 2015). Lastly, L-lactate uptake *via* MCTs is accompanied by the cotransport of protons, causing intracellular acidification (Nedergaard and Goldman, 1993), which can modulate brain energy metabolism by inhibiting phosphofructokinase (PFK), a glycolytic enzyme extremely sensitive to small changes in pH (Dienel, 2012b), and potentially other nearby ion channels, transporters, and receptors.

Extracellular L-Lactate Signaling in the Brain

Some actions of L-lactate cannot be attributed to its intracellular signaling activity, but can only be explained by L-lactate acting extracellularly as a signaling molecule. Increases in extracellular L-lactate levels that occur in the brain in response to (1) increased brain activity, (2) low oxygen availability, both triggering glycolysis and L-lactate release from neural cells, and/or (3) increased blood L-lactate levels (Boumezbeur et al., 2010; Mosienko et al., 2015) were linked to various cellular responses in the brain. Increased extracellular L-lactate due to low oxygen levels in the brain hinders prostaglandin E_2 (PGE_2) clearance, a known vasodilator, from the extracellular space by affecting prostaglandin transporter efficacy. Consequently, PGE2 concentration in the extracellular space increases, resulting in vasodilation (Gordon et al., 2008). Moreover, in rat hypothalamic vasopressin neurons, extracellular L-lactate was shown to potentiate the activity of acid-sensing ion channels (ASICs), voltage-insensitive cationic channels activated by extracellular acidification. In these neurons, L-lactate (15 mM), through chelation of extracellular Ca^{2+}, which competes with H^+ at the activation site of ASICs, increases the sensitivity of ASICs to H^+ leading to enhanced acid-induced currents (Ohbuchi et al., 2010). Various recent studies suggest that extracellular L-lactate can also activate LLRS on neural cells (**Table 1**), causing

activation of glucose and lipid metabolism in primary cortical astrocytes, modulation of neuronal activity of primary cortical neurons, and release of noradrenaline from noradrenergic neurons (Bozzo et al., 2013; Lauritzen et al., 2014; Tang et al., 2014; Mosienko et al., 2018; Vardjan et al., 2018; D'Adamo et al., 2021).

L-Lactate is a weak agonist of the $G_{i/o}$-protein coupled hydroxycarboxylic acid receptor 1 (HCAR1 or HCA1; EC_{50} of 1–5 mM; Liu et al., 2009), formerly known as orphan G-protein coupled receptor 81 (GPR81). GPR81 was first discovered in adipose tissue (Cai et al., 2008; Liu et al., 2009) and later researched in various cancers and cancer cell lines (Baltazar et al., 2020) and skeletal muscle (Rooney and Trayhurn, 2011). Although the expression level of GPR81 in the brain cells appears to be negligible according to the RNA sequencing databases and proteomic analysis (Zhang et al., 2014; Sharma et al., 2015; Zhang et al., 2016), GPR81 has been detected in the brain tissue by anti-GPR81 antibodies (Lauritzen et al., 2014). According to this study, cerebral GPR81 is concentrated predominantly in the postsynaptic membranes of the excitatory neuronal synapses, but can also be found, although to a much lesser extent, at the perisynaptic astroglial processes and at the blood-brain barrier, in particular in endothelial cells and perivascular astrocytic processes (Lauritzen et al., 2014). Consistent with the studies using anti-GPR81 antibodies, quantitative RT-PCR experiments confirmed the expression of GPR81 in mouse brain, including the cerebellum, hippocampus, and cerebral cortex (Lauritzen et al., 2014), and in isolated rat and mouse cortical astrocytes (Vardjan et al., 2018). Although under physiologic conditions, extracellular concentrations of L-lactate, that are fluctuating between the sub- and low millimolar range (0.1–1.4 mM) in rodent brain and around 5 mM in human brain, as measured by microdialysis in different brain areas (Abi-Saab et al., 2002; Mosienko et al., 2015), might be too low to fully activate GPR81, brain extracellular L-lactate concentrations can increase to several millimolar (Mosienko et al., 2015) under certain (patho)physiologic conditions, which could activate GPR81. For instance, (1) during exercise, when L-lactate blood levels increase and L-lactate enters the brain from the systemic circulation (usually the L-lactate concentration in the brain is lower than in the circulation; Bergersen, 2015), as GPR81-mediated effects of exercise on brain function were demonstrated in mice that have been subjected to high-intensity interval exercise or L-lactate injection mimicking exercise-induced increase in blood L-lactate levels (Morland et al., 2017); (2)

TABLE 1 | Astroglial and neuronal L-lactate-sensitive receptors and their effects on intracellular signaling and metabolism.

Receptor	G-protein coupling	L-Lactate sensitivity	Intracellular signaling		Metabolic effects		Other agonists	References
			Astrocytes	Neurons	Astrocytes	Neurons		
GPR81 (HCAR1)	G_i	~4–30 mM	n.d.	↓Ca^{2+}-transient frequency	n.d.	n.d.	3,5-DHBA α-HBA Glycolate γ-HBA 3Cl-5OH-BA Compound 2	Liu et al., 2009, 2012; Dvorak et al., 2012; Bozzo et al., 2013; Lauritzen et al., 2014; Sakurai et al., 2014
Neuronal LLRx	G_s	0.5 mM	n.d.	↑$[cAMP]_i$ ↑PKA activity	n.d.	n.d.	D-Lactate (antagonist) MPA aHIBA HMBA 2HPA KA	Tang et al., 2014; Mosienko et al., 2018
Astroglial unidentified LLR	G_s	20 mM	↑$[cAMP]_i$ ↑PKA activity	n.d.	↓$[glucose]_i$ ↑$[lactate]_i$ ↑lipid droplet accumulation	n.d.	3Cl-5OH-BA Compound 2	Vardjan et al., 2018; D'Adamo et al., 2021; Smolič et al., 2021
Olfr78* (OR51E2)	G_s	~4 mM	/	n.d.	/	n.d.	Acetate Propionate	Conzelmann et al., 2000; Chang et al., 2015; Mosienko et al., 2018
GPR4	Presumable allosteric modulation	1–10 mM	n.d.	n.d.	n.d.	n.d.	H^+	Hosford et al., 2018

GPR81, G-protein coupled receptor 81; HCAR1, hydroxycarboxylic acid receptor 1; LLR, L-lactate-sensitive receptor; Olfr78, L-lactate-sensitive olfactory receptor 78; OR51E2, human orthologue of L-lactate-sensitive olfactory receptor 78; GPR4, proton-sensitive G-protein coupled receptor 4; 3,5-DHBA, 3,5-dihydroxybenzoic acid; α-HBA, α-hydroxybutyrate; γ-HBA, γ-hydroxybutyrate; 3Cl-5OH-BA, 3-chloro-5-hydroxybenzoic acid; Compound 2, 2,4-methyl-N-(5-(2-(4-methylpiperazin-1-yl)-2-oxoethyl)-4-(2-thienyl)-1,3-thiazol-2-yl) cyclohexanecarboxamide; MPA, (S)-(−)-2-methoxypropionic acid; aHIBA, α-hydroxyisobutyric acid; HMBA, 2-hydroxy-3-methyl-butyric acid; 2HPA, (S)-2-hydroxypentanoic acid; KA, kynurenic acid; PKA, protein kinase A; $[cAMP]_i$, intracellular concentration of cAMP; $[glucose]_i$, intracellular concentration of free D-glucose; $[lactate]_i$, intracellular concentration of L-lactate; and n.d., not determined.

**Not expressed in astrocytes.*

when oxygen or glucose supplies in the brain are low (e.g., during hypoxia, ischemia, seizures, and hyperglycemia; Smith et al., 1986; During et al., 1994; Lee et al., 2015; Mosienko et al., 2015); and (3) when the gene expression profile of neural cells is changed favoring L-lactate production/accumulation (Afridi et al., 2020). Moreover, one can speculate that under physiologic conditions in response to increased neuronal activity, L-lactate is released from neural cells locally, in microdomains. In microdomains, the L-lactate concentration is likely high enough to fully activate GPR81, however, the presence of such microdomains in the brain needs to be determined in the future (Morland et al., 2015; Mosienko et al., 2015).

Activation of the GPR81 in adipose tissue through $G_{i/o}$-proteins downregulates the formation of cAMP. This leads to the inhibition of lipolysis, promoting lipid storage in adipocytes (Ahmed et al., 2010). Similar to adipocytes, GPR81 activation in cancer and muscle cells decreases cAMP levels (Sun et al., 2016; Feng et al., 2017), which is crucial for cancer cell survival (Roland et al., 2014) and maintenance of mitochondrial function (Sun et al., 2016; Baltazar et al., 2020), respectively. Consistent with these results, in rat hippocampal slices, increase in forskolin-induced cAMP was inhibited by L-lactate at concentrations >10 mM and by selective GPR81 receptor agonist 3,5-dihydroxybenzoic acid (3,5-DHBA) with half maximal inhibitory concentration (IC_{50}) of 1.4 mM, as measured by cAMP radioimmunoassay on homogenized brain slices (Lauritzen et al., 2014). Moreover, L-lactate (in a concentration-dependent manner with IC_{50} of ~4.2 mM) and selective GPR81 agonist 3,5-DHBA (1 mM) decreased the spontaneous electrical activity of isolated mouse cortical neurons measured as a decrease in Ca^{2+}-transient frequency. This most likely occurs *via* G_i-protein activation given that pertussis toxin, an inhibitor of G_i-proteins, prevented the decrease of neuronal activity by L-lactate (Bozzo et al., 2013), suggesting that brain GPR81 is also coupled to $G_{i/o}$-proteins and responds only to supraphysiologic L-lactate concentrations (Lauritzen et al., 2014).

Recently, it has been proposed that noradrenergic neurons (Tang et al., 2014; Mosienko et al., 2018) and cortical astrocytes (Vardjan et al., 2018; D'Adamo et al., 2021) may respond to extracellular L-lactate through as yet unidentified LLRs that are coupled to G_s-proteins and cAMP production, which is discussed in more detail in the following section.

RECEPTOR-MEDIATED L-LACTATE SIGNALING IN ASTROCYTES

Astrocytes, although electrically silent cells, can respond to many, if not all, signaling molecules in the brain (e.g., glutamate, ATP, noradrenaline, GABA, acetylcholine, serotonin, dopamine, cannabinoid, and bradykinin) through metabotropic G-protein coupled receptors (GPCRs) expressed on their surface. Activation of astroglial GPCRs can change intracellular Ca^{2+} and/or cAMP signals (i.e., cytoplasmic excitability) *via* receptor coupling to G_q- and/or G_s- and $G_{i/o}$-proteins, respectively (Vardjan and Zorec, 2015), which affects astrocyte function and control of brain homeostasis (Verkhratsky and Nedergaard, 2018). Recently, extracellular L-lactate was identified as a novel signaling molecule in the brain that could excite L-lactate-sensitive GPCRs. Initially, GPR81 receptor, coupled to $G_{i/o}$-proteins and downregulation of cAMP production, was suggested to be involved in L-lactate signaling in astrocytes (Lauritzen et al., 2014), but recently, another as yet unidentified GPCR, most likely coupled to G_s-proteins and upregulation of cAMP production, has been linked to L-lactate-mediated signaling in astrocytes (Vardjan et al., 2018; D'Adamo et al., 2021).

Intracellular Ca^{2+} and cAMP imaging of rat cortical astrocytes revealed that Ca^{2+} signals in astrocytes, preloaded with a Ca^{2+} indicator Fluo-4 AM (D'Adamo et al., 2021), are not affected by extracellular L-lactate (20 mM) and a selective GPR81 agonist, 3-chloro-5-hydroxybenzoic acid (3Cl-5OH-BA; 0.5 mM; Vardjan et al., 2018; D'Adamo et al., 2021), while both agonists trigger a persistent increase in intracellular cAMP and protein kinase A (PKA) activity in astrocytes. The latter occurs within ~100 s (cAMP) and ~200 s (PKA) upon stimulation, as measured by genetically encoded fluorescence resonance energy transfer (FRET)-based cAMP sensor Epac1-camps and a cAMP-dependent PKA activity sensor AKAR2 (Vardjan et al., 2018; D'Adamo et al., 2021). The L-lactate-induced increase in cAMP depends on the activity of transmembrane adenylate cyclase (AC; **Figure 1**), given that the treatment of cells with an AC inhibitor 2',5'-dideoxyadenosine (DDA; 100 μM) reduced the 20 mM L-lactate-induced increase in cAMP levels by ~50%, consistent with a G_s-protein signaling mechanism. Surprisingly, 3Cl-5OH-BA (0.5 mM) and a high-affinity GPR81 agonist, 2,4-methyl-N-(5-(2-(4-methylpiperazin-1-yl)-2-oxoethyl)-4-(2-thienyl)-1,3-thiazol-2-yl) cyclohexanecarboxamide (Compound 2; 50 nM; Sakurai et al., 2014) also trigger increases in cAMP in cortical astrocytes isolated from GPR81 knockout (KO) mice (Vardjan et al., 2018), indicating that L-lactate-triggered cAMP increases in astrocytes are independent of GPR81 receptor activation (Vardjan et al., 2018). These data obtained by real-time fluorescence microscopy contrast with the results obtained on adult rat hippocampal slices, where downregulation of cAMP production upon stimulation of tissue with extracellular L-lactate and selective GPR81 agonist 3,5-DHBA was linked to the activation of brain GPR81. In hippocampal slices, L-lactate and GPR81 agonist 3,5-DHBA exhibited concentration-dependent inhibition of forskolin-stimulated cAMP production with IC_{50} of ~29 mM and 1.4 mM, respectively. However, the brain cell type responsible for the observed downregulation of cAMP signals in hippocampal slices was not identified, as cAMP content was determined on homogenized tissue samples containing all brain cells (Lauritzen et al., 2014), suggesting that brain cells other than astrocytes, which express only low amounts of GPR81 (Lauritzen et al., 2014; Zhang et al., 2014; Sharma et al., 2015; Zhang et al., 2016), are responsible for L-lactate-mediated downregulation of cAMP generation in hippocampal slices.

Thus, astrocytes, in addition to GPR81, most likely express another, yet unidentified LLR coupled to G_s-proteins and AC-mediated cAMP production, which is also activated by GPR81 agonists and responds with increases in cAMP only

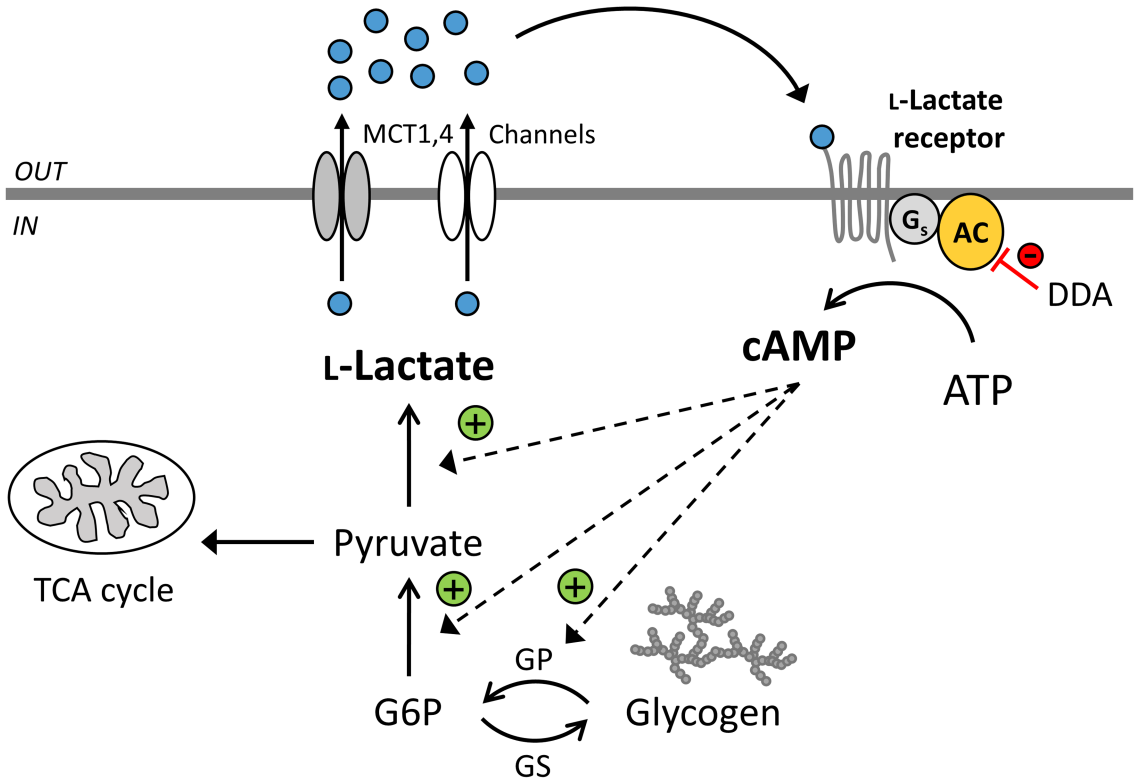

FIGURE 1 | Extracellular L-lactate enhances cytosolic L-lactate production *via* yet unidentified receptors coupled to adenylate cyclase (AC) activity and cAMP signaling in astrocytes. L-Lactate (blue circles) is formed in astrocytes (IN) in the process of aerobic glycolysis and released through monocarboxylate transporters (MCTs) 1,4 and/or L-lactate-permeable channels. Extracellularly (OUT), L-lactate can be transported to neighboring cells as a fuel, it can exit the brain *via* the circulation, or act as a signaling molecule. By binding to the L-lactate-sensitive receptors (LLRs) on the surface of astrocytes, it can stimulate AC and cAMP production. This triggers glycogen degradation, glycolysis, and more L-lactate production. The inhibition of AC by 2',5'-dideoxyadenosine (DDA) causes a reduction in the astroglial LLR-mediated increase in cAMP and L-lactate levels (red line). L-Lactate-positive feedback mechanism ("metabolic excitability") in astrocytes may maintain the L-lactate tissue concentration gradient between astrocytes and neighboring cells, enhancing the availability of L-lactate as a metabolic fuel when brain energy demands are high. DDA, 2',5'-dideoxyadenosine, an inhibitor of AC; TCA cycle, tricarboxylic acid cycle; G6P, glucose 6-phosphate; GP, glycogen phosphorylase; and GS, glycogen synthase. Channels denote lactate-permeable K^+/voltage-sensitive cation channels, pannexin and connexin hemichannels.

to supraphysiologic (20 mM) extracellular L-lactate concentrations (**Table 1**), given that 2 mM extracellular L-lactate concentration did not affect intracellular cAMP signals in astrocytes (Vardjan et al., 2018; D'Adamo et al., 2021). Interestingly, astroglial sensitivity to L-lactate-mediated cAMP elevation was increased in *Gdi1* KO cortical astrocytes isolated from a mouse model of *GDI1*-associated XLID (D'Adamo et al., 2021), a form of neurodevelopmental disorder characterized by "pure" mental deficiency (Curie et al., 2009). *GDI1* encodes for αGDI (Rab GDP dissociation inhibitor alpha) protein which regulates the GDP/GTP exchange reaction of most Rab proteins that are associated with vesicle traffic of molecules between cellular organelles (Stenmark, 2009). Namely, in *Gdi1* KO astrocytes, but not *Gdi1* WT astrocytes, extracellular L-lactate triggered intracellular cAMP increases already at a physiologic L-lactate concentration of 2 mM (D'Adamo et al., 2021), possibly due to altered expression level of the astroglial LLR and/or downstream signaling factors, which may contribute to the metabolic imbalance and disease in this form of neurodevelopmental disorder (D'Adamo et al., 2021).

Consistent with the results obtained on rat and mouse isolated cortical astrocytes, the existence of a neuronal LLR that activates AC and cAMP production, named LLRx, was proposed in *locus coeruleus* (LC) noradrenergic neurons, which can, in contrast to an astroglial LLR, respond to physiologic extracellular L-lactate concentrations (**Table 1**; Tang et al., 2014; Mosienko et al., 2018). Studies performed on brainstem organotypic-cultured slices from rat pups containing LC noradrenergic neurons showed that exogenously applied L-lactate (2 mM), as well as L-lactate released from astrocytes in response

to optogenetic excitation, trigger depolarizations in noradrenergic neurons and subsequent release of stress response neuromodulator noradrenaline. The latter was suppressed if the slices were treated with oxamate (20 mM), an L-lactate synthesis inhibitor, or 1,4-dideoxy-1,4-imino-d-arabinitol (DAB; 500 μM), a glycogen shunt activity inhibitor, implying that astroglial-derived L-lactate is involved in activation of noradrenergic neurons. Moreover, treatment of slices with AC inhibitor SQ22536 (100 μM) and PKA inhibitor H89 (10 μM) suppressed the depolarizing effect of 2 mM L-lactate, indicating involvement of G_s-protein coupled receptors and the cAMP/PKA signaling pathway in L-lactate-mediated activation of noradrenergic neurons. The authors also propose that noradrenaline released from noradrenergic neurons can then back-excite neighboring astrocytes (Tang et al., 2014; Mosienko et al., 2018), most likely *via* astroglial adrenergic receptors (Bekar et al., 2008; Hertz et al., 2010; O'Donnell et al., 2012; Ding et al., 2013; Vardjan et al., 2014; Horvat et al., 2016), which may affect astrocyte function, including glucose metabolism, which is highly regulated in the brain by the activity of noradrenergic neurons (Bélanger et al., 2011; O'Donnell et al., 2012; Gibbs, 2015; Dienel and Cruz, 2016; Vardjan and Zorec, 2017; Bak et al., 2018).

So far, two G_s-coupled LLRs were identified in the brain: olfactory receptor Olfr78 (human ortholog OR51E2) in mouse olfactory sensory neurons in certain brain areas (i.e., brainstem and nucleus tractus solitarius; Conzelmann et al., 2000) and GPR4 expressed in neurons in various rodent brain areas, such as retrotrapezoid and raphe nuclei, rostral ventrolateral medulla, septum, and LC (**Table 1**; Mosienko et al., 2017; Hosford et al., 2018; Mosienko et al., 2018). According to the RNA sequencing database (Zhang et al., 2014), Olfr78 and GPR4 expression in astrocytes is negligible, and most likely does not contribute to the observed L-lactate-induced increases in cAMP signals in astrocytes.

EXTRACELLULAR L-LACTATE AND CONTROL OF ASTROGLIAL AEROBIC GLYCOLYSIS

Astrocytes are key neural cells controlling metabolic homeostasis of the brain (Verkhratsky and Nedergaard, 2018). Due to their specific glycolytic profile, they are the main site of L-lactate production and release and an almost exclusive store of glycogen in the brain, which they can rapidly mobilize to enter the glycolytic pathway (Magistretti and Allaman, 2015; Oz et al., 2015; Bak et al., 2018).

As a response to increased neuronal activity, astrocytes upregulate glucose metabolism, i.e., glucose uptake from the circulation, glycogenolysis, and aerobic glycolysis with L-lactate production (Pellerin and Magistretti, 1994; Hertz et al., 2015; Dienel and Cruz, 2016). Astrocytes can sense neuronal activity *via* changes in extracellular K^+ (Bittner et al., 2011; Sotelo-Hitschfeld et al., 2015), and glutamate levels (Pellerin and Magistretti, 1994), both tightly coupled with Na^+ fluxes across the membrane (Bittner et al., 2011; Chatton et al., 2016; Rose and Verkhratsky, 2016). Astrocytes respond to local increase in extracellular K^+ with plasma membrane depolarization leading to an increase in intracellular pH, mediated by an electrogenic Na^+/HCO_3^- cotransporter (NBCe1), which stimulates aerobic glycolysis (Ruminot et al., 2011), most likely through activation of PFK, a pH-sensitive glycolytic enzyme (Dienel, 2012b) and/or HCO_3^--mediated activation of soluble AC (Choi et al., 2012). On the other hand, extracellular glutamate stimulates astroglial aerobic glycolysis to provide energy for the activity of the Na^+/K^+ ATPase pump, which is activated by an increase in the intracellular concentration of Na^+ due to Na^+-glutamate cotransport into astrocytes (Pellerin and Magistretti, 1994, 1996; Bittner et al., 2011). Active neurons also release NH_4^+, a by-product of catabolism, which in astrocytes causes an increase in intracellular L-lactate concentration *in vitro* and *in vivo*. However, the effect of NH_4^+ on L-lactate production is not due to glycolytic stimulation, instead it affects mitochondrial pyruvate shunting by diverting the flux of pyruvate from mitochondria to L-lactate production (Lerchundi et al., 2015). Moreover, astrocytes can also sense increased neuronal activity *via* signaling molecules released from activated neurons. The latter bind to astroglial metabotropic GPCRs and ionotropic receptors (Verkhratsky and Nedergaard, 2018), which leads to intracellular increases in Ca^{2+} and/or cAMP signals in astrocytes (Vardjan and Zorec, 2015). In astrocytes, both Ca^{2+} and cAMP can increase glycogenolysis and aerobic glycolysis with L-lactate production (Horvat et al., 2021). Astrocytes are also active participants in the neurovascular unit where they can respond to nitric oxide (NO) released by endothelial cells. NO inhibits astrocytic respiration and stimulates aerobic glycolysis, resulting in glucose depletion and L-lactate production *via* inhibition of mitochondrial cytochrome oxidase (Almeida et al., 2004; San Martín et al., 2017) and increased activity of 6-phosphofructo-1-kinase (PFK1), a master regulator of glycolysis (Almeida et al., 2004).

L-Lactate is not only produced but also released from astrocytes into the extracellular space *via* plasmalemmal lactate transporters MCT1 and 4, putative K^+/voltage-sensitive cation channels (Sotelo-Hitschfeld et al., 2015), and/or hemichannels (Karagiannis et al., 2016). The presence of plasmalemmal L-lactate transporters in astrocytes and other brain cells represents the basis for a flux of L-lactate along its concentration gradient from astrocytes to (1) other brain cells, most importantly neurons (Pellerin and Magistretti, 1994, 2012) or (2) the circulation to exit the brain (Dienel, 2012a). According to the astrocyte-neuron L-lactate shuttle (ANLS) hypothesis, astroglial-derived extracellular L-lactate is taken up by neurons *via* the MCT2 transporters and fuels neuronal oxidative metabolism (Pellerin and Magistretti, 1994, 2012; Mächler et al., 2016), especially when energy demands are high, which is particularly important during memory formation and consolidation (Newman et al., 2011; Suzuki et al., 2011; Hertz et al., 2013; Descalzi et al., 2019; Murphy-Royal et al., 2020). Despite growing evidence supporting the ANLS hypothesis, the research community is not unanimous on this topic (Dienel, 2017, 2019). Some data challenge this hypothesis by showing that during brain activation neurons in acute mouse hippocampal brain slices and *in vivo* rely on their own L-lactate production rather than L-lactate derived from

astrocytes to meet the increased energy demands, while ANLS may possibly function at rest, as studied by real-time two-photon fluorescence lifetime imaging microscopy (FLIM) of NADH dynamics (Díaz-García et al., 2017). One of the main arguments based on which the existence of an ANLS hypothesis has been questioned is that most of the past work supporting this process has been performed on primary astrocytes and neurons. However, recently ANLS was described by real-time two-photon FRET microscopy and L-lactate nanosensor also *in vivo* in mice (Mächler et al., 2016) and *Drosophila* (Liu et al., 2017) and was shown to be impaired in the *in vivo* mice models of Alzheimer's disease (Sun et al., 2020).

Astroglial-derived extracellular L-lactate may also act extracellularly as a signaling molecule, activating LLRs on the surface of neural cells as discussed in the previous section (**Table 1**; Tang et al., 2014; Mosienko et al., 2015, 2018; Vardjan et al., 2018; D'Adamo et al., 2021). L-Lactate released from astrocytes was shown to excite noradrenergic neurons *via* AC-mediated cAMP signaling to release noradrenaline, which can then back-excite astrocytes (Tang et al., 2014). Activation of astroglial α_1- and β-adrenergic receptors and intracellular Ca^{2+} and cAMP signals by noradrenaline is known to upregulate glucose uptake, glycogenolysis, and aerobic glycolysis in astrocytes, which can lead to more L-lactate production and release (Sorg and Magistretti, 1991; Gibbs, 2015; Horvat et al., 2017; Vardjan et al., 2018; Velebit et al., 2020; Fink et al., 2021). Moreover, extracellular L-lactate (20 mM), as well as GPR81 agonist 3Cl-5OH-BA (0.5 mM), were shown to upregulate aerobic glycolysis and L-lactate production in isolated cortical astrocytes as measured by Laconic, a FRET-based lactate nanosensor (Vardjan et al., 2018; D'Adamo et al., 2021). 3Cl-5OH-BA-induced L-lactate production in astrocytes was greatly reduced in the presence of AC inhibitor DDA (100 μM), suggesting the involvement of a G_s-protein coupled LLR and cAMP signals in the regulation of L-lactate-induced aerobic glycolysis in astrocytes (Vardjan et al., 2018; D'Adamo et al., 2021; **Figure 1**).

Compared with noradrenaline (200 μM), which increases cytosolic free D-glucose concentration *via* activation of α_1-adrenergic receptors and Ca^{2+} signaling due to Ca^{2+}-driven extracellular D-glucose uptake (Prebil et al., 2011; Vardjan et al., 2018; D'Adamo et al., 2021; Horvat et al., 2021), L-lactate (20 mM) and 3Cl-5OH-BA (0.5 mM) decrease cytosolic free D-glucose in astrocytes, as measured with FLII^{12}Pglu-700μδ6, a genetically encoded FRET-based glucose nanosensor (**Table 1**; Vardjan et al., 2018). This is consistent with the fact that extracellular free D-glucose uptake depends primarily on Ca^{2+} signals but not cAMP signals (Horvat et al., 2021), and the fact that extracellular L-lactate and GPR81 agonists trigger increases in cAMP signals but not Ca^{2+} signals in astrocytes (Vardjan et al., 2018; D'Adamo et al., 2021). The observed decrease in cytosolic D-glucose levels in astrocytes exposed to extracellular L-lactate or 3Cl-5OH-BA most likely indicates entry of free D-glucose into the glycolytic pathway.

Thus, L-lactate released from astrocytes may not only activate neurons but may also act in an autocrine manner augmenting its own production in astrocytes. This new positive feedback mechanism of receptor-mediated L-lactate signaling ("metabolic excitability"; Vardjan et al., 2018) that controls astroglial L-lactate production may serve to maintain high intracellular levels of L-lactate, facilitating L-lactate release and thereby generating a concentration gradient between astrocytes and neurons to provide a continuous source of L-lactate to support neural network activity (Mächler et al., 2016; **Figure 1**). However, because relatively high concentrations of L-lactate (20 mM) are needed to facilitate cAMP-mediated aerobic glycolysis (Vardjan et al., 2018; D'Adamo et al., 2021), the new putative astroglial excitatory LLR mechanisms may be particularly relevant under supraphysiologic and pathologic conditions (i.e., ischemia and epilepsy; During et al., 1994; Mosienko et al., 2015), during exercise (Matsui et al., 2017), or at the sites of local extracellular L-lactate increases (L-lactate production in microdomains), if they exist, which needs to be studied in the future (Bergersen and Gjedde, 2012).

L-LACTATE AND THE CONTROL OF BRAIN LIPID METABOLISM

Regulation of brain glucose metabolism has been in the spotlight of the research community for a long time, but the importance of lipid metabolism in brain function has only gained attention in recent years (Panov et al., 2014).

Glial-neuronal coupling of glucose and lipid metabolism was recently suggested to occur as a response to neural activity to protect neurons from lipotoxicity (Liu et al., 2017; Ioannou et al., 2019). The mechanism proposes that L-lactate transport from astrocytes to neurons *via* ANLS triggers *de novo* synthesis of free fatty acids (FFAs) from L-lactate in stressed overstimulated neurons. L-Lactate is decarboxylated in neuronal mitochondria and the resulting acetyl-CoA generates FFAs. Excess of FFAs in neurons is associated with the lipid peroxidation chain reaction and generation of reactive oxygen species (ROS), which may lead to lipotoxicity. To avoid lipotoxicity, excess FFAs are considered to be transferred from neurons to glial cells, particularly astrocytes, in vesicles containing apolipoprotein E-like particles, where they are stored in lipid droplets (LDs; Liu et al., 2015, 2017; Ioannou et al., 2019). FFAs stored in LDs can be used by astrocytes as an energy substrate in β-oxidation (Ioannou et al., 2019), because astrocytes have the capacity to fight mitochondrial overproduction of ROS during β-oxidation. Recently, it was shown that chronic (24 h) exposure of both tissue astrocytes and isolated cortical astrocytes in the absence of neurons to 20 mM extracellular L-lactate (Smolič et al., 2021) triggers LD accumulation in astrocytes (**Table 1**). This suggests the existence of an alternative L-lactate-mediated mechanism augmenting LD accumulation in astrocytes. Extracellular L-lactate could affect LD turnover in astrocytes by entering cells *via* MCTs and/or ion channels (Sotelo-Hitschfeld et al., 2015), where L-lactate acts as a substrate for *de novo* FFA synthesis, as shown in oligodendrocytes (Sánchez-Abarca et al., 2001) and neurons (Liu et al., 2017; Ioannou et al., 2019), leading to excess FFA production and FFA storage in LDs to protect astrocytes from lipotoxicity. But extracellular

L-lactate may also trigger LD accumulation in astrocytes through actions *via* plasmalemmal LLRs, which needs to be investigated in more detail in the future.

CONCLUSION AND PERSPECTIVES

In conclusion, it is now well established that multiple brain functions are either supported or modulated by L-lactate acting either as a metabolic substrate or signaling molecule. The discovery of signaling properties of L-lactate in astrocytes that are manifested as upregulation in intracellular cAMP production, suggests the existence of a new, as yet unidentified, L-lactate sensitive GPCR coupled to G_s-proteins in astrocytes. L-Lactate-triggered cAMP signals in astrocytes facilitate aerobic glycolysis with more L-lactate production (metabolic excitability), likely to provide neurons with more L-lactate. Moreover, chronic exposure to L-lactate triggers accumulation of LDs in astrocytes, suggesting that astrocytes switch to lipid metabolism. However, this is achieved only at relatively high extracellular concentrations of L-lactate, implying a role of L-lactate signaling in astrocytes particularly at the sites of putative L-lactate microdomains, under pathologic conditions, or during exercise. Identification of L-lactate-sensitive GPCRs in astrocytes and increasing knowledge on this topic will provide further insights into our understanding of the importance of L-lactate signals in the regulation of brain metabolism and support of brain performance.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Lactate Is a Metabolic Mediator That Shapes Immune Cell Fate and Function

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Lactate and the associated H^+ ions are still introduced in many biochemistry and general biology textbooks and courses as a metabolic by-product within fast or oxygen-independent glycolysis. However, the role of lactate as a fuel source has been well-appreciated in the field of physiology, and the role of lactate as a metabolic feedback regulator and distinct signaling molecule is beginning to gain traction in the field of immunology. We now know that while lactate and the associated H^+ ions are generally immunosuppressive negative regulators, there are cell, receptor, mediator, and microenvironment-specific effects that augment T helper (Th)17, macrophage (M)2, tumor-associated macrophage, and neutrophil functions. Moreover, we are beginning to uncover how lactate and H^+ utilize different transporters and signaling cascades in various immune cell types. These immunomodulatory effects may have a substantial impact in cancer, sepsis, autoimmunity, wound healing, and other immunomodulatory conditions with elevated lactate levels. In this article, we summarize the known effects of lactate and H^+ on immune cells to hypothesize potential explanations for the divergent inflammatory vs. anti-inflammatory effects.

Keywords: lactic acid, lactate, immunosuppression, Th17, immunometabolism, M2, immune, inflammation

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INTRODUCTION

The field of physiology has long recognized the importance of metabolic pathways for energy sustaining adenosine triphosphate (ATP) production in homeostasis and in response to physiological stressors. However, the importance of cell metabolism within immunology has only become more appreciated in the past decade (O'Neill et al., 2016). We now understand that immune cells differentially utilize glycolysis vs. oxidative phosphorylation (OX PHOS) for differentiation, polarization, and effector functions (Norata et al., 2015; Loftus and Finlay, 2016). Moreover, we are just beginning to understand the preferential use of different substrates and the full functionality of different metabolites.

Lactate, once considered a metabolic waste product, is not only produced during glycolytic ATP production, but can be used for energy production, gluconeogenesis, and autocrine, paracrine, and endocrine signaling (Gladden, 2004; Brooks, 2018). In 1985, the cell-to-cell lactate shuttle theory introduced the idea that lactate can be produced in one [muscle] cell type and consumed in another (Brooks, 1985, 2009). We now know this to be true in the

immune system as well. Lactate is elevated in inflammatory diseases due to increased production or impaired clearance, which then influences immune cell function. Several recent reviews have discussed the role of lactate in immune cell activation within one specific disease context (Pucino et al., 2017; Brooks, 2018; Santos et al., 2019; Baltazar et al., 2020; Ivashkiv, 2020). Thus in the current work, we have reviewed multiple cell types and disease models to help shape our overall understanding of how lactate influences immune cell function. Importantly, we offer potential explanations for the seemingly contradictory pro- and anti-inflammatory functions of lactate and lactic acid.

While lactate, nicotinamide adenine dinucleotide (NAD+), and ATP are considered primary metabolic products of glycolytic metabolism, there is also a concurrent release of H^+ ions (Kemp, 2005; Kemp et al., 2006; Brooks, 2018; Qian, 2018). From an immunological perspective, both lactate and H^+ ions appear important for cellular function and feedback, as they can act separately or together to influence immune function. Thus, this review will cover immunological studies of lactate alone and lactate with the associated H^+ ions. Although lactic acid is always dissociated at physiological pH, this terminology will be specifically used when lactic acid was added to culture systems or animal models with the understanding that dissociation occurs and results are due to both ions (see specific experimental details described in **Supplementary Table 1** and below).

RECEPTOR TRANSPORT AND METABOLISM

There are multiple mechanisms by which lactate and the associated H^+ ions can enter immune cells. Proton-dependent monocarboxylate transporters (MCTs) are the primary proteins known to facilitate H^+-dependent transport of monocarboxylates, such as lactate, down their concentration gradients (Halestrap, 2012, 2013; Sun et al., 2017). MCT-1 (Slc15a) is the primary lactate importer, and MCT-4 (Slc16a3) is the primary lactate exporter, both with ubiquitous cell expression (Halestrap, 2012; Contreras-Baeza et al., 2019). MCT-1 has been shown to mediate the effects of lactate and the associated H^+ ions in macrophages, mast cells, and $CD8^+$ T cells (Colegio et al., 2014; Haas et al., 2015; Abebayehu et al., 2016; Caslin et al., 2019; Zhang et al., 2019b).

In addition to transport *via* MCT-1, lactate can also be transported through sodium-dependent transporters (Slc5a12) on $CD4^+$ T cells (Haas et al., 2015), and can activate G-protein coupled receptor (GPR)81 on monocytes, macrophages, and dendritic cells (Hoque et al., 2014; Brown et al., 2020). Moreover, tissue acidification by H^+ ions is sensed by GPR65 and GPR132 on macrophages (Chen et al., 2017; Bohn et al., 2018). These receptors transport different substrates and have selective expression on immune cells (Haas et al., 2015), which has been proposed to orchestrate differential functional responses by different immune cell types (Pucino et al., 2017).

Once inside the cell, a buildup of lactate and the associated H^+ ions generally act as negative feedback regulators for glycolytic ATP production. H^+ ions inhibit phosphofructokinase activity (Dobson et al., 1986; Leite et al., 2011), and lactate is converted to pyruvate by lactate dehydrogenase, which impairs NADH recycling (Gray et al., 2014; Angelin et al., 2017). Because NAD^+ is necessary for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) function, NAD^+ is needed for sustained glycolysis (Quinn et al., 2020). Lactate and the associated H^+ ions have similar inhibitory effects on glycolysis in immune cells (**Figure 1**). Lactic acid, which rapidly dissociates to lactate and H^+, suppressed glycolysis in the myeloid lineage and T cells, as measured by Seahorse metabolic flux analysis (Dietl et al., 2010; Errea et al., 2016; Ratter et al., 2018; Fischbeck et al., 2020; Quinn et al., 2020). Lactic acid suppressed glucose uptake, lactate export, glycolytic enzyme expression, and intracellular ATP levels in monocytes and mast cells (Dietl et al., 2010; Caslin et al., 2019). Further, exogenous lactate reduced the NAD^+/NADH ratio in activated $CD4^+$ T cells, suggesting NADH-to-NAD^+ recycling was inhibited, leaving less available NAD^+ for the continuation of glycolysis (Pucino et al., 2019). Impaired NADH recycling also limited serine production, which was important for T cell activation (Quinn et al., 2020).

In the past 10–15 years, immunologists have begun to fully appreciate how bioenergetic pathways are linked with inflammatory function. Generally, glycolysis fuels inflammatory cells and oxidative phosphorylation supports anti-inflammatory, regulatory cells (O'Neill et al., 2016). This suggests that lactic acid-mediated glycolytic inhibition may suppress inflammatory immune cell function and promote regulatory functions.

IMMUNE EFFECTS AND SIGNALING MECHANISMS

Many publications support the idea that lactic acid and lactate are generally immunosuppressive. However, more recent publications suggest that cell, receptor, and microenvironmental effects also determine how lactate and lactic acid influence inflammatory macrophage, neutrophil, and T helper (Th)17 cell function. Lactic acid not only inhibits glycolytic energy production, but additional mechanisms of action occur *via* changes in signaling cascades and epigenetic modifications. In this section, we will summarize the literature by cell type, describe the mechanisms of action, and discuss potential explanations for seemingly opposing findings. Please see **Figure 2** for a general summary of the effects of lactate and lactic acid on immune cell subsets, **Supplementary Table 1** for specific study details and findings, and **Figure 1** for signaling cascades that contribute to the effects of lactic acid on immune function in specific cell types.

Myeloid Immune Cells

Monocytes and Macrophages

The effects of lactic acid and lactate have been most studied on innate myeloid cells. In monocytes and macrophages, lactic acid suppressed an array of lipopolysaccharide (LPS)-induced cytokine and chemokine mediators (Dietl et al., 2010; Peter et al., 2015; Errea et al., 2016). Lactate itself also suppressed

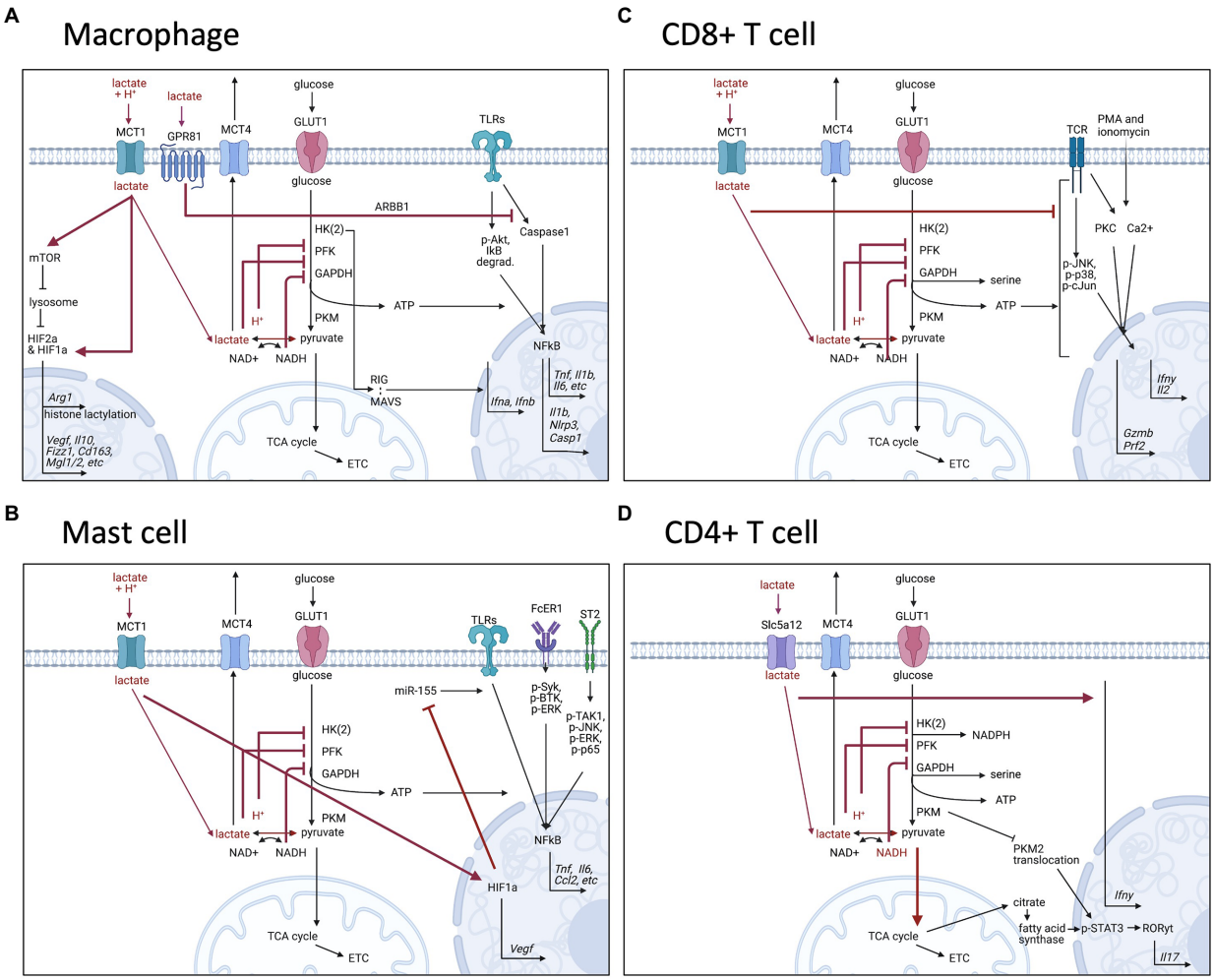

FIGURE 1 | Signaling mechanisms of lactate by cell type. **(A)** Monocytes and macrophages, **(B)** Mast cells, **(C)** $CD8^+$ T cells, and **(D)** $CD4^+$ T cells. Figure created with BioRender.com.

inflammasome assembly, LPS-stimulated cytokine secretion, and migration in macrophages and monocytes (Goetze, 2011; Hoque et al., 2014; Ratter et al., 2018). Interestingly, Dietl et al. (2010) found the effects of lactic acid on human monocytes to be pH-dependent, which is supported by two studies on acidity in alveolar macrophages (Bidani, et al., 1998; Fernandez et al., 2013). Conversely, Peter et al. (2015) found lactic acid, lactate, and acidity to have differential effects depending on the gene of interest. Thus, both lactate and the associated H^+ ion may influence macrophage function.

Lactic acid and lactate can suppress not only glycolysis, but specific receptor signaling cascades in macrophages and monocytes (see **Figure 1A**). The mechanisms of action are varied. For example, lactic acid inhibited LPS receptor signaling by delaying protein kinase B (AKT) phosphorylation, inhibitor of nuclear factor kappa B (IκB-alpha) degradation, and nuclear factor kappa B (NFκB) nuclear accumulation and activation (Hoque et al., 2014; Peter et al., 2015). Lactate has also impaired toll-like receptor (TLR)-4 mediated inflammasome assembly by eliciting GPR81-induced inhibitory signals (Hoque et al., 2014; Zhang et al., 2019b). Also, lactate can directly interact with the mitochondrial antiviral-signaling (MAVS) protein, preventing MAVS aggregation and therefore reducing type I interferon production during RIG-I-like receptor (RLR) signaling (Zhang et al., 2019b). Together these data show the variety of mechanisms by which lactate and the associated H^+ ion can influence macrophage activation.

Lactic acid not only suppresses inflammatory macrophage (M1) function; it enhances regulatory, or anti-inflammatory, M2 polarization. Multiple publications have shown that lactic acid induced M2-associated genes (Colegio et al., 2014; Bohn et al., 2018; Zhang and Li, 2020). This polarization was dependent on MCT transport, hypoxia inducible factor (HIF) activation, and inducible cyclicAMP early repressor (ICER) induction (Colegio et al., 2014; Bohn et al., 2018; Liu et al., 2019; Zhang and Li, 2020). HIFs are transcription factors known to regulate both metabolic and inflammatory genes, and ICER is a transcriptional repressor that inhibits TLR-dependent NFκB

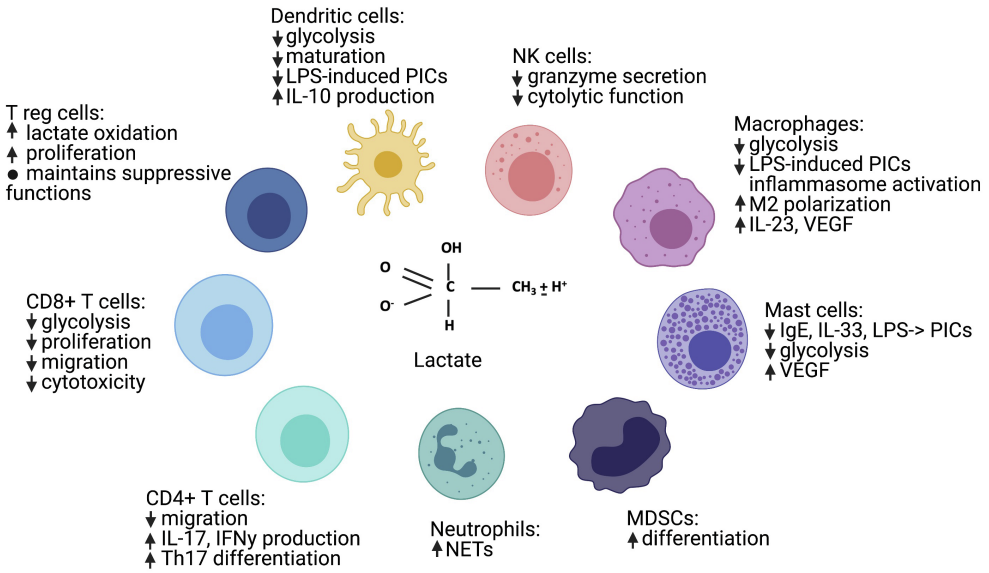

FIGURE 2 | Lactate has many immune cell-specific effects, ranging from general immunosuppression to Th17 and M2 polarization. "↑" indicates a positive signal or induction, "↓" indicates a negative signal or inhibition, and "PICs" indicates "pro-inflammatory cytokines." Figure created with BioRender.com.

signaling (Corcoran and O'Neill, 2016; Bohn et al., 2018). These data suggest that there are multiple regulatory control points for lactate within signaling cascades and gene transcription that contribute to observed effects. Additionally, arginase *(Arg)-1* transcription has been induced following histone lactylation, a lysine modification that occurs 16–24 h following M1 activation (Zhang et al., 2019a). Thus, lactate and the associated H^+ ions appear to be an intrinsic regulatory feedback pathway to help reduce macrophage inflammation and restore homeostasis.

In contrast to the above immunosuppressive effects, stimulatory effects of lactic acid and lactate on myeloid cell populations have also been reported. Lactic acid has been repeatedly shown to increase LPS-induced IL-23 production in monocytes, macrophages, and tumor infiltrating immune cells (Shime et al., 2008; Witkin et al., 2011; Peter et al., 2015). Moreover, lactate has been shown to increase prostaglandin E_2 (PGE2) synthesis (Wei et al., 2015). Together, these data suggest a mediator-specific effect. However, lactate has also been shown to enhance the LPS-induced secretion of IL-6, matrix metalloproteinase (MMP)1, and IL-1β from U397 cell lines and human monocyte-derived macrophages (Nareika et al., 2005; Samuvel et al., 2009) and to increase NFκB activity *via* MCTs (Samuvel et al., 2009), which is in direct contrast to the above publications. It is not entirely clear why these publications found inflammatory effects of lactic acid, while others repeatedly reported anti-inflammatory effects. Both the Nareika and Samuvel publications out of the Huang group used U397 cell lines, which are derived from a histiocytic lymphoma, and thus may have intrinsic characteristics that differ from murine and human monocytes and macrophages (Nareika et al., 2005; Samuvel et al., 2009). Samuvel et al. (2009) also assessed human monocytes-derived macrophages, and it is possible that the observed effects may have been influenced by *ex vivo* differentiation or culture conditions—such as the inclusion of insulin and glutamine in culture media.

This hypothesis that the microenvironment may influence the effects of lactate is supported by two additional studies in the tumor immunology field. When monocytes were differentiated in the presence of lactate with granulocyte-macrophage colony-stimulating factor (GM-CSF), or adenocarcinoma-conditioned media (with elevated lactate and many other mediators), they increased both inflammatory (M1) and regulatory (M2) mediators, consistent with a tumor-associated macrophage (TAM) phenotype (Penny et al., 2016; Paolini et al., 2020). Further experimentation showed that GM-CSF and lactate together drove IL-6-dependent macrophage colony-stimulating factor (M-CSF) production and consumption, which promoted an inflammatory feed-forward loop. It is plausible that other factors in the tumor-conditioned media also had a similar impact. Thus, these two studies suggest that soluble mediators in the microenvironment may influence lactate effects, especially during differentiation.

Dendritic Cells and Myeloid-Derived Suppressor Cells

Similar to the inhibitory effects reported in monocytes and macrophages, exogenous and endogenous lactic acid reduced dendritic cell maturation and suppressed LPS-induced cytokine production (Gottfried, 2006; Nasi et al., 2013). Tumor-derived lactate also activated the receptor GPR81 on dendritic cells to reduce antigen presentation, cytokine production, and cyclic adenosine monophosphate (cAMP) activation (Brown et al., 2020). Additionally, tumor-derived lactic acid may also augment T helper (Th)2 polarization by dendritic cells (Selleri et al., 2016). This further supports the M2

polarization data discussed above and suggests that lactic acid may promote a general Th2/M2 regulatory response. Furthermore, lactate augmented differentiation of myeloid-derived suppressor cells (Husain et al., 2013), providing more support for a general inhibitory effect in myeloid cells.

Mast Cells

Recent publications from our laboratory have extended these findings to mast cells. We have shown that lactic acid suppresses immunoglobulin (Ig)E- and IL-33-dependent inflammatory cytokine and chemokine production (Abebayehu et al., 2016, 2019). Moreover, lactic acid suppressed inflammatory cytokine and chemokine production following TLR activation by bacterial and viral stimuli (Caslin et al., 2019). Interestingly, lactic acid also increased the secretion of the angiogenic factor vascular endothelial growth factor (VEGF) following IgE and IL-33 activation, supporting the reported macrophage M2 polarization data above. The suppressive effects of lactic acid in mast cells were dependent on MCT-1, and were reproduced by acidification or high concentrations of lactate (Abebayehu et al., 2016; Caslin et al., 2019). Additionally, inhibiting glycolysis mimicked lactic acid, while increasing ATP availability reversed lactic acid effects on LPS signaling (Caslin et al., 2019). These latter data suggest that suppressing glycolytic ATP production may be necessary and sufficient for lactic acid effects.

Our data also support and extend the signaling cascades and epigenetic mechanisms by which lactic acid can suppress myeloid activation (see **Figure 1B**). Similar to macrophages, we found that lactic acid reduced NFκB activity downstream of LPS (Caslin et al., 2019) and promoted HIF-1α-dependent VEGF production (Abebayehu et al., 2016, 2019). We also reported that lactic acid inhibits kinase activation downstream of the IL-33 and IgE receptors. In addition, lactic acid reduced microRNA (miR)-155-5p expression following IL-33 and LPS activation. miR-155 diminishes expression of inhibitory proteins (Huffaker and O'Connell, 2015; Lind et al., 2015), and thus amplifies inflammatory signaling. In our studies, a miR-155-5p mimic reversed the suppressive effects of lactic acid, indicating lactic acid can preserve negative feedback pathways partly by suppressing miR-155-5p. Interestingly, lactic acid still inhibited LPS-induced cytokine production in miR-155 knockout mice, suggesting that lactic acid may act by multiple redundant mechanisms.

In another recent publication, lactic acid suppressed calcium mobilization, degranulation, and the release of chemokines and cytokines through the MAS-related GPR family member X2 (MRGPRX2) receptor in culture (Syed et al., 2021). Lactic acid also reduced passive systemic anaphylaxis to compound 48/80 and skin inflammation in a mouse model of rosacea. Together with our results, these data suggest that lactic acid suppresses mast cell-mediated inflammation.

Neutrophils

The effects of lactate have also been studied in neutrophils. Interestingly, endogenous and exogenous lactate treatment induced neutrophil extracellular trap (NET) formation (Awasthi et al., 2019), an inflammatory function aiding bacterial and viral clearance. The mechanisms by which lactate induced NET formation are not known. As glycolysis is needed for NET production (Rodríguez-Espinosa et al., 2015), these results may seem surprising. However, the formation of superoxide as a reactive oxygen species can also induce NET formation (Al-Khafaji et al., 2016), and glycolytic inhibition promotes oxidative stress in many cell types (Le et al., 2010; Korga et al., 2019). The relative contribution of glycolysis and oxidative stress to NET formation and lactate action may be an interesting area of study for the future research. Importantly, these findings further suggest that lactate effects are cell-specific and can be pro-inflammatory.

Lymphoid Immune Cells

Natural Killer Cells and Cytotoxic ($CD8^+$) T Cells

In the lymphoid compartment, the effects of lactic acid and lactate have been studied in NK and T cells. In NK cells, lactic acid (both lactate and acidity) inhibited cytolytic function (Husain et al., 2013). In $CD8^+$ T cells, lactic acid reduced proliferation, degranulation, motility, cytolytic activity, and inflammatory mediator secretion (interferon (IFN)γ, perforin, and granzyme; Fischer et al., 2007; Mendler et al., 2012; Haas et al., 2015; Fischbeck et al., 2020). In $CD8^+$ T cells, these effects are likely due to altered receptor signaling, as lactic acid suppressed protein kinase activity (Mendler et al., 2012), and suppression was dependent on MCT-1 (Haas et al., 2015; for signaling, see **Figure 1C**). Interestingly, glycolytic suppression was not shown to be required for all lactic acid effects in $CD8^+$ T cells (Haas et al., 2015), suggesting that other mechanisms of action beyond metabolism—such as cell signaling—should be explored.

Helper ($CD4^+$) T Cells: T Regulatory and T Helper 17 Cells

In the $CD4^+$ T cell lineage, lactic acid and lactate have differential effects. Lactate suppressed $CD4^+$ cell motility while increasing IL-2 secretion (Roth and Droge, 1991; Haas et al., 2015), which can promote T cell differentiation, including that of T regulatory cells (Treg; Chinen et al., 2016). Tregs have also been shown to uptake and metabolize lactate to maintain suppressor function in high lactate conditions, like the tumor microenvironment (Watson et al., 2021). Specifically, Tregs converted lactate into pyruvate, citrate, and malate to fuel the tricarboxylic acid (TCA) cycle and into phosphoenolpyruvate for glycolytic intermediates essential for proliferation. This could logically extend the findings of lactic acid-mediated suppression of NK and $CD8^+$ T cells, because Tregs also inhibit NK- and $CD8^+$ T cell activity (Plitas and Rudensky, 2016).

Despite promoting Treg-mediated inhibition, lactate can promote the differentiation of naive $CD4^+$ T cells into inflammatory Th17 cells (for signaling, see **Figure 1D**). Two recent studies suggest that sodium lactate increased *Il17* and *Ifn*γ gene expression *via* retinoic acid receptor-related orphan receptor gamma (RORγT), suggesting Th17 polarization (Haas et al., 2015; Pucino et al., 2019). Unlike $CD8^+$ cells, which express MCT-1, $CD4^+$ T cells express Scl5a12, a sodium-coupled lactate transporter, which may explain the opposite

effects of lactic acid and lactate on inflammatory function in these cell types (Haas et al., 2015). Through Slc5a12, lactate not only blunted glycolytic energy production, but increased oxidative stress, promoting the translocation of pyruvate kinase into the nucleus to phosphorylate signal transducer and activator of transcription (STAT3/1), and inducing RORγt-dependent IL-17 transcription (Pucino et al., 2019). Interestingly, lactate-induced IL-17 production was also due to fatty acid synthesis *via* the pentose phosphate pathway, nicotinamide adenine dinucleotide phosphate (NADPH), and TCA-derived citrate, suggesting the involvement and coordination of many metabolic pathways. These studies add considerable detail to understanding how lactate interacts with metabolism to alter cellular function. It is not yet clear how lactate controls both Treg suppressor functions and inflammatory Th17 differentiation; however, there is a unique relationship between both cell types (reviewed in DeBerardinis et al., 2007). Treg and Th17 differentiation both require TGFβ, which can induce T cells expressing the lineage-defining transcription factors for both Treg (Foxp3) and (RORγt). Cytokines in the microenvironment drive further differentiation, with IL-2 inducing Tregs and IL-6 and IL-21 inducing Th17 cells. Moreover, Tregs and Th17 can trans-differentiate in some situations. Different utilization of bioenergetic pathways, like the hexosamine pathway, and different metabolite-induced epigenetic landscapes appear to contribute to the development of Treg vs. Th17 cells (DeBerardinis et al., 2007). However, the role of lactate is poorly understood. Future studies should consider how lactate promotes the development of each cell type and the role of the microenvironment in shaping that response.

Discussion of Cell-Specific Effects

The opposing pro- and anti-inflammatory effects of lactate with and without the associated H^+ ions have several possible explanations. For example, the divergent effects on $CD8^+$ and $CD4^+$ T cells appear due to selective MCT-1 vs. Scl5a12 expression, respectively (Haas et al., 2015). It is possible that transporter diversity also explains other divergent findings. Lactic acid suppression was MCT-1 dependent in many myeloid populations; however, receptor dependency was not measured in all studies. It would be particularly interesting to know which receptors are required for myeloid cell IL-23 induction, the mixed tumor-associated macrophage phenotype, and enhanced neutrophil NET formation.

In addition to transporter expression, acidity has been linked to differential effects. Some studies reported that lactic acid effects are pH-dependent (Dietl et al., 2010), while others have found the effects of lactic acid, lactate alone, and acidity specific to the gene of interest (Peter et al., 2015). We hypothesize that some of these differential effects are due to substrate concentrations and receptor kinetics. The predominant role of MCT transporters is proton-linked transport of L-lactate, but at higher concentrations, MCT can exchange one monocarboxylate molecule for another without net proton movement (Halestrap, 2012). In our studies, 12.5-mM lactic acid suppressed cytokine production and reduced the pH of the buffered media to 6.7 (Caslin et al., 2019). It remained significantly lower than control media for 1 h. Fischbeck et al. (2020) observed a similar drop in pH with 10-mM lactic acid and a drop to pH 6.3 with 20-mM lactic acid addition. Additionally, we observed that formic acid, with a similar pKa, also suppressed IL-6 secretion, and sodium lactate above 20 mM could mimic lactic acid. The roles of acidity and concentration are supported in dendritic cells, where the inhibitory effect of 10-mM lactic acid was reversed by adjusting the pH to 7.4, but adjusting the pH had less effect at concentrations above 10 mM (Gottfried, 2006). Moreover, sodium lactate suppressed LPS-induced cytokine production, but only when present for a prolonged period of time (Ratter et al., 2018), whereas we found that lactic acid could suppress cytokine production when added simultaneously, or even after activation (Caslin et al., 2019). These data suggest that in addition to differential transporter expression, concentration and acidity can alter the outcome using the same transporter. Lactate and the associated H^+ ions can act at lower concentrations, while lactate alone can mimic these effects when present at higher concentrations. Future studies should more thoroughly investigate the effects of lactate vs. pH as well as the receptor utilized by each cell type.

Another set of seemingly contradictory results is the influence of lactate with and without the associated H^+ ions on HIF-1α and HIF-2α. These transcription factors are commonly upregulated in hypoxic environments to enhance glucose and iron metabolism, angiogenesis, and erythropoiesis. In myeloid cells, HIF-1α generally promotes glycolysis, increases pro-inflammatory gene expression, and mediates bacterial killing (Imtiyaz and Simon, 2010). Distinct from these hypoxia-induced effects, our laboratory and others have shown that lactic acid induces HIF-1α function while suppressing glycolysis and inflammatory cytokine production (Colegio et al., 2014; Abebayehu et al., 2016, 2019). The selective induction of HIF-1α-dependent VEGF production suggests specific transcriptional effects. Furthermore, HIF-1α has been shown to promote Th17 polarization (Shi et al., 2011; Corcoran and O'Neill, 2016). Thus, a lactate-HIF connection appears to control multiple axes of inflammation and angiogenesis. HIFs are controlled at multiple levels, with both transcriptional and post-translational regulation, with additional environmental factors like iron availability influencing HIF degradation (Siegert et al., 2015). Thus, lactate and associated H^+ ions may access multiple levels of HIF regulation and could offer insight into how these important transcription factors control inflammation and angiogenesis.

Finally, the biological rationale for promoting Th17, Th2, and M2 responses is unclear and likely represents an opportunity to better understand fundamental aspects of immunity. Lactic acid induces both Th17 differentiation and myeloid IL-23 production (which promotes Th17 differentiation; Shime et al., 2008; Haas et al., 2015; Pucino et al., 2019). In apparent contrast to this, lactic acid not only induces M2 polarization (Colegio et al., 2014; Bohn et al., 2018; Zhang and Li, 2020), but also augments dendritic cell-mediated Th2 polarization (Selleri et al., 2016). Th17 and Th2/M2 responses are often seen as acting in opposition. However,

recent studies suggest that Th17 responses are crucial for intestinal hypercontractility and worm expulsion in anti-helminth immunity (Allen et al., 2015; Steel et al., 2019), which traditionally requires Th2 responses. Moreover, IL-17- and Th2-type cytokines can enhance or counter-regulate each other in the response to helminths (Allen et al., 2015). Adding another layer to this model system, lactic acid also suppresses mast cell IgE- and IL-33-induced responses (Abebayehu et al., 2016, 2019), which contribute to anti-helminth immunity. Induction of both Th17 and Th2/M2, in addition to suppressing mast cells, suggests either cooperativity between these branches of immunity, an attempt to maintain homeostasis, or perhaps both at different periods of the infection. Future studies should examine the contribution of cell, receptor, mediator, and microenvironment-specific effects, and should aim to uncover a more complete understanding of the biological role of lactic acid on the different types of immunity.

IMPLICATIONS FOR DISEASE

Lactate is elevated in many disease states, either systemically or locally, *via* enhanced production and/or impaired clearance. Lactate is produced and consumed by many different cell types, and the effects of lactate on a variety of cell types likely play a role in disease pathogenesis and prognosis. We will primarily focus here on the immune-specific effects of lactate and lactic acid, with an effort to differentiate lactate and pH effects. Some studies have injected lactic acid into animal models, and H+ ions are likely produced alongside lactate in many animal models. The pH effects can be buffered by bicarbonate or other molecules, but this varies with the organ involved. We hypothesize that lactate is a negative feedback regulator in acute inflammatory conditions. However, it is important to remember that by definition, chronic disease states represent a loss of homeostasis, making it difficult to untangle cause and consequence from association.

Cancer

In the 1920s, it was initially shown that tumor cells consumed substantial glucose and secreted lactate even in the presence of adequate oxygen (Cori and Cori, 1925; Warburg et al., 1927). This is now known as the Warburg effect (Potter et al., 2016), and is a hallmark characteristic of cancer (Hanahan and Weinberg, 2011). By making ATP from glycolysis, cancer cells can use metabolic intermediates from other pathways for proliferation (Potter et al., 2016). In the tumor microenvironment, lactate levels can reach 40 mM (Ostroukhova et al., 2012), with extracellular pH as low as 6–6.5 (Wike-Hooley et al., 1984). This is a striking comparison to normal cell environments with a lactate level ~ 1 mM and pH of 7.2. Additionally, high lactate levels are associated with increased metastasis and decreased survival (Walenta et al., 2000, 2004), suggesting that lactate may be used as a clinical prognostic parameter.

Lactate can not only provide a survival advantage for tumor cells by upregulating oncogenes and inducing angiogenesis; it promotes immune evasion and is considered an oncometabolite (Choi et al., 2013; San-Millán and Brooks, 2017; San-Millán et al., 2019). Lactic acid from the tumor microenvironment suppressed $CD8^+$T cell activation and tumor killing (Fischbeck et al., 2020). Additionally, lactic acid promoted both M2 differentiation and a mixed M1/M2 macrophage phenotype characteristic of tumor-associated macrophages that aid in immune escape (Colegio et al., 2014; Helm et al., 2014; Penny et al., 2016; Mu et al., 2018; de-Brito et al., 2020; Paolini et al., 2020). Lactate and lactic acid also augmented HIF-1α-mediated VEGF production from mast cells (Abebayehu et al., 2016, 2019) and macrophages (Colegio et al., 2014) which may contribute to angiogenesis and tumor growth. Moreover, lactate enhanced the synthesis of prostaglandin E2 (PGE2) by cyclooxygenase (COX)2 in monocytes, which is involved in tumor progression and the development of therapeutic resistance (Wei et al., 2015; Tong et al., 2018). Together, these immunosuppressive effects select for tumor growth and escape, migration, invasion, and immune evasion (Choi et al., 2013; Biswas, 2015; Mu et al., 2018). Interestingly, many known chemotherapeutic agents are weak bases, whose ionization in the acidic tumor environment reduces uptake and efficacy (Raghunand et al., 1999; Sun et al., 2017).

There have been a few approaches *in vivo* to directly target lactic acid effects on tumor growth and immune function. Bicarbonate added to drinking water has been shown to reduce melanoma tumor size, increase tumor-associated $CD8^+$ cells, and enhance survival in mice (Pilon-Thomas et al., 2016). Combining bicarbonate therapy with immunotherapy for melanoma or doxorubicin treatment for breast cancer appears to augment drug effects (Raghunand et al., 1999; Pilon-Thomas et al., 2016). Furthermore, proton pump inhibitors, which can increase tumor pH, significantly increased survival and T cell function in a murine melanoma model (Calcinotto et al., 2012). Finally, diclofenac, a lactate dehydrogenase (LDH)A inhibitor, has been used in a murine glioma model to reduce lactic acid secretion, effectively enhancing DC inflammatory capacity and reducing the accumulation of Tregs (Chirasani et al., 2013). However, the therapy also suppressed T cell glycolysis, compromising IFN-γ production and T-cell proliferation. This highlights the importance of glycolysis for immune cell function and suggests therapeutics should specifically target lactate consumption or signaling to enhance immune function, instead of targeting lactate production. Preventing lactate actions on T cells and macrophages without suppressing glycolysis should enhance anti-tumor immunity.

Wound Healing

Lactate levels are also elevated in the local wound environment, typically reported around 20 mM, with a range between 5 and 80 mM (Löffler et al., 2011; Britland et al., 2012). This is due to poor tissue perfusion, poor oxygenation, or atypical bacterial colonization and immune activation (Britland et al., 2012). As stated, lactate induced M2 polarization and VEGF production (Constant et al., 2000; Trabold et al., 2003; Hunt et al., 2007; Porporato et al., 2012; Colegio et al., 2014; Abebayehu et al., 2016, 2019). In contrast to cancer, these effects are beneficial

and promote angiogenesis, endothelial cell migration, and wound closure (Hunt et al., 2007; Porporato et al., 2012). Additionally, lactate enhanced fibroblast proliferation, myofibroblast differentiation, and collagen deposition (Trabold et al., 2003; Wagner et al., 2004; Kottmann et al., 2012), which also contribute to wound healing.

Local and systemic lactate delivery *via* lactate-releasing polymers can promote angiogenesis, endothelial progenitor cell recruitment, procollagen activation, and extracellular matrix deposition in mice with ischemic wounds (Trabold et al., 2003; Porporato et al., 2012). Interestingly, poly(lactic-co-glycolic acid; PLGA) nanoparticle delivery of VEGF can accelerate non-diabetic and diabetic wound healing faster than either VEGF or PLGA lactate-based polymers alone (Chereddy et al., 2015). While these therapeutics are promising, further understanding of the mechanisms by which lactate and the associated H^+ ions can induce immune resolution and repair may help develop even better wound treatments.

Sepsis

Sepsis is a pathological inflammatory response to systemic infection. One hallmark is elevated lactate levels due to tissue hypoperfusion, impaired pyruvate dehydrogenase activity, elevated catecholamine secretion, and increased immune cell activation (Vary, 1996; Haji-Michael et al., 1999; McCarter et al., 2001). Blood lactate concentrations in sepsis are often between 2 and 10 mM; however, concentrations have been reported as high as 20 mM due to the timing of measurement and severity of disease (Filho et al., 2016; Kuttab et al., 2018; Theerawit et al., 2018). Many studies show that elevated blood lactate (≥4 mM) and impaired clearance are independently associated with increased mortality in septic patients (Nguyen et al., 2004; Trzeciak et al., 2007; Arnold et al., 2009; Nichol et al., 2011; Marty et al., 2013). Thus, the use of lactate clearance as a treatment guideline for sepsis has gained traction in adult and pediatric patients alike (Rhodes et al., 2017; Kuttab et al., 2018; Nazir et al., 2019). It is not fully understood if lactate is a cause or a consequence of sepsis. As articulated by Brooks, hyperlactemia is often a strain on the system to lessen the effects of injury. However, while this may be protective during the initial cytokine storm, it can be pathological in the late stage of sepsis (Brooks, 2018; Caslin et al., 2019).

We and others reported that lactic acid and lactate suppressed LPS-induced cell metabolism and immune cell function, which may impair antibacterial defense mechanisms (Gottfried, 2006; Peter et al., 2015; Errea et al., 2016; Caslin et al., 2019). We have also shown that intraperitoneal lactic acid administration prior to LPS injection suppressed cytokine production in mice (Caslin et al., 2019). Similarly, sodium lactate suppressed cytokine production in a rat model of sepsis (Besnier et al., 2020). Intratracheal acidic aspiration has also been shown to impair clearance of *S. pneumoniae* and *E. coli* (Fernandez et al., 2013). Together with the clinical observations above, these data support the theory that high lactate levels early in sepsis may act to suppress immune cell glycolysis and function. However, this negative regulation is detrimental, impairing pathogen clearance, and contributing to immunosuppression observed in the secondary phase of sepsis. This latter phase is marked by reduced glucose metabolism, cytokine production, antigen presentation, and cytolytic function (Hotchkiss et al., 2013a,b), and thus resembles the effects of lactate and lactic acid treatment. Separate from this general suppressive activity, the ability of lactate or lactic acid to augment NET formation and IL-17 production, while impairing $CD8^+$ degranulation and mobilization (Haas et al., 2015; Awasthi et al., 2019; Pucino et al., 2019), may also explain how the septic cytokine storm can be disconnected from bacterial clearance. Future studies should directly examine the effects of early lactate clearance on *ex vivo* immune cell metabolism and function in septic patients during the immunosuppressive phase of the disease. Additionally, future studies should attempt to reconcile these potentially detrimental immune effects with the seemingly beneficial effects of lactate metabolism on other organ systems (Garcia-Alvarez et al., 2014; Hernandez et al., 2019; See and Bellomo, 2021). Lactate may be increased *via* oxidation and gluconeogenesis to maintain organ function. Thus, future therapeutics could not only attempt to reduce lactate uptake or signaling in immune cells, but aim to selectively increase oxidation in non-immune cells.

Ulcerative Colitis and Rheumatoid Arthritis

Lactate levels are elevated in many autoimmune diseases, including ulcerative colitis and rheumatoid arthritis. In patients with ulcerative colitis, those with moderate and severe colitis have low fecal pH and high fecal lactate, produced by inflamed colonic mucosal cells (Vernia et al., 1988; Hove et al., 1995). In an experimental mouse model of colitis, knocking out the lactate receptor GPR81 increased inflammatory cytokine production in intestinal dendritic cells and macrophages and worsened colonic inflammation (Ranganathan et al., 2018). Pharmacological activation of GPR81 reduced colonic inflammation. Similarly, lactate administration prior to murine colitis onset reduced inflammation and serum IL-6 (Iraporda et al., 2016). Together, these data suggest that lactate may act as a negative feedback regulator limiting colitis inflammation.

Arthritic joints have also long been recognized as a site of high lactate (Goetzi et al., 1971; Treuhaft and McCarty, 1971), due to rapid synovial fibroblast turnover and proliferation (Pucino et al., 2019). Lactate transporter expression in the synovia correlated with T cell number in rheumatoid arthritis patients (Haas et al., 2015). Moreover, in contrast to healthy T cells, naive $CD4^+$ T cells from rheumatoid arthritis patients were unable to upregulate the glycolytic enzyme PFKFB3, resulting in a state-of-energy deprivation and senescence (Pucino et al., 2019). These results suggest that lactate may contribute to T cell dysfunction in arthritis. More research should be done to understand how lactate levels contribute to disease progression in autoimmunity.

Asthma and Allergic Disease

Systemic elevations in lactate have also been reported in asthma and allergic disease. Elevated plasma lactate has been measured in asthmatic patients, which correlated with reduced pulmonary function measured by forced expiratory volume in 1 s (FEV1; Ostroukhova et al., 2012). These elevations were only ~1 mM

above normal serum levels; however, patients with stable asthma, rhinitis, and eczema also have lower lung pH as measured by exhaled breath condensate (EBC) than controls, and acute asthmatics have even lower EBC pH compared to the other groups (Brunetti et al., 2006). One potential explanation for the association between lactate and asthmatic severity is that patients with severe and steroid resistant asthma often have elevated Th2-driven inflammation and Th17-driven neutrophilic infiltration (Newcomb and Peebles, 2013; Irvin et al., 2014), both of which are augmented by lactate and the associated H^+ ions.

However, lactic acid suppressed both IgE and IL-33-induced mast cell activation (Abebayehu et al., 2016, 2019), which should logically improve allergic disease. These effects occurred in culture, but also in a model of IL-33-induced peritonitis and IgE-driven passive anaphylaxis. Moreover, lactic acid-producing *lactobacilli* probiotic strains have been shown to improve asthma and allergic disease (Żukiewicz-Sobczak et al., 2014), although these results could be attributed to the tissue microbiome and additional immunomodulatory metabolites like butyrate. Thus, asthma is another example of cell-specific lactic acid effects that are poorly understood and need to be unraveled to move beyond disease association.

Obesity

Patients with obesity and diabetes have higher plasma lactate concentrations than healthy volunteers (Chen et al., 1993); however, there is little known about the role of lactate in obesity. Adipocytes regularly produce lactate, which increases with adipocyte size, cell density (*ex vivo*), and epinephrine or insulin stimulation (Digirolamo et al., 1992; Krycer et al., 2020). Fat cells from obese or diabetic rats (or humans) can metabolize lactate to as much as 50–70% of imported glucose. Additionally, lactate affects cell redox, beta-oxidation, and lipolysis in adipocytes (Brooks, 2018). These complex effects again show that cell-specific lactate effects must be uncovered to understand and target pathways in disease.

Interestingly, while lactate production from lean adipocytes could logically support resident M2-like adipose tissue macrophages, obesity is associated with inflammatory macrophages that undergo glycolysis and also produce lactate (Caslin and Hasty, 2019). These macrophages have been shown to promote insulin resistance and promote diabetes (Lumeng et al., 2007). This contradiction between lactate production and inflammatory phenotype is poorly understood, and it remains plausible that metabolism influences their role in adipose tissue (O'Neill et al., 2016). Interestingly, these cells are similar to some tumor-associated macrophages (Helm et al., 2014; de-Brito et al., 2020); in that obese adipose tissue macrophages increased both M1- and M2-like markers and increased both glycolytic and oxidative metabolism, suggesting a mixed phenotype (Kratz et al., 2014; Boutens et al., 2018; Li et al., 2019). As lactate is elevated in both the tumor microenvironment and adipose tissue, future studies should explore the role of lactate in the unique polarization status of macrophages in these environments. Both tumor-associated macrophages and obese adipose macrophages have roles in lipid and iron handling (Kratz et al., 2014; Orr et al., 2014; Jung et al., 2017; Su et al., 2020), suggesting microenvironmental stimuli may modulate cell polarization and function. Like sepsis and asthma, we do not know the extent to which lactate is beneficial vs. pathological in obesity or how lactate acts in obesity-related diabetes.

The above should demonstrate the variety of diseases affected by elevated lactate levels, but we would like to point out that this is not a comprehensive list. Lactate therapy has been proposed for diseases like pancreatitis (Wu et al., 2011; Hoque et al., 2014) and myocardial infarction (Zhang et al., 2021). In many disease models, increasing buffering capacity or modulating lactate transporter expression may improve disease outcomes (Raghunand et al., 1999; Chirasani et al., 2013; Ohashi et al., 2013; Pilon-Thomas et al., 2016; Feichtinger and Lang, 2019; Pucino et al., 2019). Many studies have also attempted to administer probiotic lactobacilli strains, lactate producing polymers, or lactate infusions (Herías et al., 2005; Wu et al., 2011; Żukiewicz-Sobczak et al., 2014; Iraporda et al., 2016). Together, these emphasize that understanding the effects of lactate on inflammation may be critical and fruitful for many disease states.

CONCLUSION

Immunologists are just now beginning to understand the role of lactate and the associated H^+ ions as metabolites, feedback regulators, and signaling molecules within the immune system and more broadly within physiology. Lactate and H^+ ions generally act to suppress glycolytic ATP production, contributing to reduced inflammatory cell signaling and mediator production. However, lactate with and without the associated H^+ ions also have specific receptor-mediated functions and can promote Th2 and Th17 immunity. Additionally, lactate and the associated H^+ ions appear to promote a mixed M1/M2 phenotype in tumor-associated macrophages, suggesting a potential role for additional signaling mediators in the microenvironment.

The implications for some of the seemingly contradictory effects of lactate with and without the associated H^+ ions are not clear. Studies suggest that Th2 and Th17 responses may coordinate in anti-helminth immune defense, suggesting an evolutionary role for promoting both responses. However, the suppressive effects of lactate and the associated H^+ ions on mast cell activation complicate this hypothesis. Moreover, lactate is elevated in many disease conditions and appears to have both detrimental and beneficial effects. Following acute immune cell activation, such as in wound healing, lactic acid may act as a negative feedback regulator. In chronic inflammation, elevated lactate levels may represent an inability to control inflammation or respond to feedback.

While many murine studies have attempted to modulate lactate and the associated H^+ ions, there is still much to unravel about the contribution of cell, receptor, mediator, and microenvironment effects. It would be useful for researchers to further investigate the specific role of lactate vs. H^+ in

each immune cell subset, as well as the contribution of intracellular vs. extracellular (paracrine/endocrine) lactate production. Additionally, it would be useful to further clarify the contribution of lactate production and clearance to each disease and the role of lactate in both immune and non-immune cell types. If lactate oxidation is beneficial for one cell type yet detrimental for another, targeting specific cell types or specific signaling mechanisms will be important. Finally, future studies should further explore the mechanisms by which lactate and the associated H^+ ions suppress Th1-mediated inflammation while promoting M2- and Th17-driven responses, which may help to identify and develop more effective therapeutic targets for diseases like cancer, sepsis, allergic diseases, and autoimmune diseases. Thus, while we still have much to learn, it is evident that lactate and the associated H^+ ions have systemic influence on not only the immune system, but physiology in health and disease.

AUTHOR CONTRIBUTIONS

HC was invited to write the review and wrote the initial manuscript draft, while all authors contributed to idea development and manuscript edits. Moreover, HC, DA, and JR contributed to previous publications from the Ryan Lab which sparked our interest and understanding of this topic. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.688485/full#supplementary-material

Supplementary Table 1 | Detailed findings reported in lactate-immune literature. Effect on inflammation noted by color: blue = decreases, orange = increases, green = both. Cell type abbreviations: PBMC, peripheral blood mononuclear cell; (i) BMDM, (immortalized) bone marrow-derived macrophage; MDDC, monocyte-derived dendritic cell; MDM, monocyte-derived macrophage; MDSC, monocyte-derived suppressor cells; BMMC, bone marrow-derived mast cells; BMDM, bone marrow-derived macrophage; BMMC, bone marrow-derived mast cells; BMDC, bone marrow-derived dendritic cells; NK, natural killer; CTL, $CD8^+$ or cytotoxic killer T cell. Treatment: H_3PO_4, phosphoric acid; HCl, hydrochloric acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; NaOH, sodium hydroxide; TCM, tumor-conditioned media. Activation signal abbreviations: BCG-CWS, Bacille Calmette-Guerin cell wall skeleton; LPS, lipoteichoic acid; PGN, peptidoglycan; LTA, lipoteichoic acid; OVA, ovalbumin; Pam3Cys, Pam3Cys-Ser-(Lys)4; Poly(I:C), polyinosinic/polycytidylic acid; PMA, phorbol myristate acetate. Effector molecules: AKT, protein kinase B; AP-1, activator protein 1; ARG1, arginase 1; ATP6V0d2, macrophage-specific V-ATPase subunit; BTK, Bruton's tyrosine kinase; CCl, chemokine ligand (C-C motif); CD, cluster of differentiation; CSF, colony stimulating factor; COX2, cyclooxygenase-2; Cxcl, chemokine ligand (C-X-C Motif); ERK, extracellular signal-regulated kinase; GEM, GTP binding protein overexpressed in skeletal muscle; GPR, G-protein coupled receptor; HIF, hypoxia inducible factor; HK, hexokinase; ICER, inducible cyclic AMP early repressor; IFN, interferon; Ig, immunoglobulin; IL-, interleukin; JNK, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; M, macrophage; MAVS, mitochondrial antiviral signaling protein; MCP, monocyte chemoattractant protein; MCT, monocarboxylic transporter; MD2, myeloid differentiation factor 2; MIP, macrophage inflammatory protein; miR, microRNA; MMP, matrix metalloprotease; mTORC, mammalian target of rapamycin complex; NFκB, nuclear factor kappa B; p, protein kinase; PGE2, prostaglandin E2; PEPCK, phosphoenolpyruvate carboxykinase; PTX3, pentraxin 3; RIG1, retinoic acid-inducible gene 1; RORγt, retinoic acid receptor-related orphan receptor gamma; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; SYK, spleen tyrosine kinase; TAK1, transforming growth factor-β-activated kinase 1; TCR, T cell receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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METHODS
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Modelling of Blood Lactate Time-Courses During Exercise and/or the Subsequent Recovery: Limitations and Few Perspectives

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Because lactate is an important metabolic intermediate and a signalling molecule between/within cells/organs, it appears essential to be able to describe the kinetics of this central molecule, during and/or after physical exercise. The present study aimed to confront three models and their approaches [Freund and co-workers (F&co), Beneke and co-workers (B&co), and Quittmann and co-workers (Q&co)] to investigate the lactate exchange (γ_1) and removal (γ_2) abilities (min^{-1}) during and/or after exercise. Nine healthy male subjects performed 3- and 6-min easy, moderate, and heavy exercise. Blood lactate concentration (BLC) was measured every 5 s over the entire period of exercise and recovery. Approaches differ depending on the domain in which the model is applied: considering exercise and part of the recovery (B&co and Q&co) or the entire period of recovery (F&co). The different approaches result in differing γ_1 and γ_2 values. Model fitting is closer to the experimental values following the method (model and approach) of F&co. Complementary analyses show that consideration of (i) exercise drastically impairs the quality of model fitting and therefore the γ_1 and γ_2 values and (ii) the entire period of recovery considerably improves the quality of fits and therefore of the γ_1 and γ_2 values. We conclude that (i) it is neither realistic nor reliable to take into account exercise and recovery in the same model and (ii) the longer the period of recovery studied, the better the quality of the γ_1 and γ_2 values.

Keywords: modelling, lactate, exercise, recovery, curves, velocity constants, kinetics

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INTRODUCTION

Muscle contraction requires energy that comes from adenosine triphosphate (ATP) hydrolysis. Because of extremely low levels of ATP stores, several metabolic pathways are activated to resynthesize it. Glycogenolysis and glycolysis are major components of these metabolic pathways. Their activation induces lactate production, which increases with exercise intensity (Stanley et al., 1985; MacRae et al., 1992). When this production cannot be balanced anymore by removal processes, lactate accumulates in muscle (Chwalbinska-Moneta et al., 1989; Juel et al., 1990) and blood (Stanley et al., 1985; MacRae et al., 1992; Bergman et al., 1999; Messonnier et al., 2013). Blood lactate profiles obtained during incremental (Davis et al., 1983; Heck et al., 1985) or constant load exercises (Heck et al., 1985; Beneke, 1995) have been well-described. Different indexes drawn from the blood lactate vs. work rate curve have shown to be closely related to performance in different

sporting activities (Faude et al., 2009) or markers of exercise intensity during endurance training in athletes (Yu et al., 2012; Tran et al., 2014) and in patients (Messonnier et al., 2021).

Lactate is well-known as a metabolic intermediate between and within cells and organs (lactate shuttle concept) for cell fuelling (Brooks, 1986, 2000, 2009) inducing and contributing to metabolic flexibility of various cells, such as cancer cells (Brooks, 2018; Brooks et al., 2021). It can also serve as a cell-signalling molecule and has the potential for the regulation of gene expression and epigenetic modifications (Hashimoto et al., 2007; Brooks, 2018; Brooks et al., 2021). From that point of view, it appears essential to be able to describe the kinetics of this central molecule, during and/or after physical exercise.

Tracer studies constitute the reference method to determine lactate kinetics parameters, such as the lactate rates of appearance (R_a) and disappearance (R_d) (Brooks et al., 1991; Miller et al., 2002; Messonnier et al., 2013). However, to be fully applicable and reliable, tracer techniques require almost steady states in blood lactate and tracer concentrations and a near equilibrium in concentrations between the compartments of the body (e.g., between active muscles and blood). These requirements are not fulfilled during high-intensity exercise and its subsequent recovery. Therefore, alternative methods should be found and applied. Freund and co-workers (F&Co) described the blood lactate profiles during recovery following short high-intensity exercises using a biexponential time function referring to a two-compartment lactate distribution space (Freund and Gendry, 1978; Freund and Zouloumian, 1981a,b; Zouloumian and Freund, 1981a,b). The interest of this approach lies in the fact that it allows for the determination of two major components of lactate kinetics, namely, the lactate exchange (γ_1) and removal (γ_2) abilities during recovery (Freund et al., 1986; Chatel et al., 2016). Of note, γ_1 and γ_2 should not be mixed up with R_a and R_d, which refer to different concepts. Using this approach, it has been shown that lactate exchange and removal abilities decrease with exercise duration and intensity (Freund et al., 1986, 1989; Chatel et al., 2016). Therefore, these abilities refer to the physiological state of the subject at the end of the exercise. Furthermore, it has been shown that these abilities were (i) improved by endurance training (Messonnier et al., 2001), (ii) different according to physical ability profiles of athletes (Bret et al., 2003), and (iii) related to performance (Messonnier et al., 1997, 2002). Applications of this model have been proposed, allowing for the estimation of net lactate release rate, the net amount of lactate released, lactate disappearance rate, and lactate metabolic clearance rate during recovery (Bret et al., 2003; Messonnier et al., 2006; Chatel et al., 2016).

The model proposed by Freund and Zouloumian and used later by their successors involved a passive and almost complete recovery, meaning that measurements of blood lactate concentrations (BLCs) during recovery may last 60–90 min if the exercise was heavy. To bypass this inconvenience, Beneke and co-workers (B&co) (Beneke et al., 2005) and more recently Quittmann and co-workers (Q&co) (Quittmann et al., 2018) proposed adapted models and approaches allowing reductions of lactate collection time during recovery to 20 and 10 min, respectively. To obtain realistic predictions and especially a return to lifelike resting blood lactate values, their models start at the pre-exercise BLC and use only one amplitude. If this approach forces the models to return to realistic resting BLC (i.e., the pre-exercise BLC), this implies that their models apply to both exercise and recovery. While the approach proposed by F&co provides information concerning lactate exchange and removal abilities during recovery, the approaches proposed by Beneke et al. (2005) and Quittmann et al. (2018) suggest that the same lactate exchange and removal abilities prevail during both exercise and recovery. This contradicts the fact that these abilities are altered by exercise itself (Freund et al., 1986, 1989). Does it constitute a pitfall?

The aim of the present study was to assess the three approaches using the same set of experimental data and compare the results. The hypothesis is that the adapted models are less precise than the models and approaches used by F&co and less universally applicable regardless of the performed exercise.

MATERIALS AND METHODS

The data set used in the present study had been obtained previously (Freund et al., 1989). The methods are repeated here for the convenience of the reader. The study was conducted according to the laws and standards at the time of the experiment.

Subjects

Nine healthy male subjects volunteered to participate in this study. Their age, weight, height, maximal oxygen uptake (VO_{2max}), and maximal aerobic power (power associated with VO_{2max}) were 21.0 ± 2.4 years old, 67.7 ± 6.4 kg, 175 ± 8 cm, 3.76 ± 0.51 L min^{-1}, and 284 ± 33 W, respectively. Prior to giving their written consent, all subjects were informed of the aim and potential risks or discomforts associated with the experiments.

Experimental Design

All exercise tests were performed on the same ergo cycle (Fleisch ergometer) at a constant pedalling frequency of 60 rpm. Prior to the experiments, subjects underwent a physical examination and an incremental exercise up to exhaustion to determine the maximal oxygen uptake (VO_{2max}) and the corresponding maximal aerobic power. On two occasions 2 weeks apart, subjects were tested in the morning 2 h after having had a light standard breakfast. Under local anaesthesia, an indwelling catheter was placed in the brachial artery. The tests were performed at the normal room temperature (21–23°C). Each experiment was performed in the following order: a rest of 30 min, an easy exercise intensity [(mean ± SEM) 1.81 ± 0.03 W·kg^{-1}; 39–50% VO_{2max}] followed by a recovery of at least 30 min, a moderate exercise intensity (2.53 ± 0.08 W·kg^{-1}; 52–67% VO_{2max}) with subsequent recovery of at least 60 min, and finally, a heavy exercise intensity (3.52 ± 0.17 W·kg^{-1}; 76–82% VO_{2max}) followed by a recovery period of at least 90 min. On the first occasion, five subjects were randomly assigned to 3-min exercises while the four remaining subjects performed 6-min exercises.

On the second occasion (2 weeks later), subjects reiterated the protocol on a crossover basis, switching from 3- to 6-min or to 6- to 3-min exercises. Each exercise and its subsequent recovery were considered separately. Seven curves were missing (three after 3-min exercises and four after 6-min exercise) due to exercises not being performed.

Arterial Blood Sampling and Analysis

To avoid coagulation, the subjects were heparinized (100 IU·kg^{-1} body mass). Throughout exercise and recovery, arterial blood was sampled via a catheter at a rate of 0.32 mL·min^{-1} by means of a well-calibrated peristaltic pump. Arterial blood was analysed automatically for lactate concentration (Freund, 1967). Lactate concentration was determined on a continuous flow custom-made analyser using an enzymatic method where the oxidation of lactate to pyruvate is coupled in the presence of lactate dehydrogenase and NAD^+ to NADH + H^+ formation. The operations required for the biochemical reactions (such as mixing, dialyses, heating and additions of buffers, lactate dehydrogenase, and NAD^+) were carried out automatically by the analyser. The changes of absorption were measured at 340 or 365 nm (5–10 nm light path). The electrical signals supplied by the colorimeter were converted in lactate concentrations by means of the two standard curves determined before and after each experiment. It is worth mentioning that the time necessary for the blood to flow through the catheter was precisely measured before each experiment. The response time of the analyser was also rigorously measured during its calibration. Both these times were accounted for the synchronisation of data with the experimental protocol. For further details, we refer the reader to previous publications (Freund, 1967; Freund et al., 1989). Total blood loss during a test amounted to 0.100–0.150 L. Signals from the biochemical analyser allowed recording of BLCs every 5 s. Experimental values of maximal blood lactate concentrations (BLC_{max}, mmol L^{-1}) and time to reach BLC_{max} ($TBLC_{max}$, min) were recorded.

Mathematical Analysis

Arterial blood lactate (exercise and/or recovery) curves were fitted by Equation 1 (Freund and co-workers), Equation 2 (Beneke and co-workers), and Equation 3 (Quittmann and co-workers) using an iterative non-linear regression technique (Kaleidagraph 3.6, Synergy Software, PA, USA).

For the convenience of the readers, the original equations have been reworded to contain consistent terminology over the three equations.

$$[La](t) = [La]_{(completion)} + A_1(1 - e^{-\gamma_1 \cdot t}) + A_2(1 - e^{-\gamma_2 \cdot t}) \quad (1)$$

$$[La](t) = [(A \cdot \gamma_1)/(\gamma_2 - \gamma_1)].(e^{-\gamma_1 \cdot t} - e^{-\gamma_2 \cdot t}) + [La]_{(0)} \quad (2)$$

$$[La](t) = [La]_{(0)} + A(1 - e^{-\gamma_1 \cdot t}) - A(1 - e^{-\gamma_2 \cdot t}) \quad (3)$$

where [La] is arterial lactate concentration (mmol·L^{-1}), $La_{(completion)}$ is the BLC at exercise completion (onset of recovery, mmol·L^{-1}), $[La]_{(0)}$ is the BLC at the beginning of exercise (resting value, mmol·L^{-1}), t is time (min), A_1 and A_2 are the amplitudes of the experimental terms (mmol·L^{-1}) describing lactate appearance and disappearance, respectively, A is this amplitude of appearance and disappearance (difference from maximal and resting BLC, mmol·L^{-1}), and γ_1 and γ_2 are the velocity constants (min^{-1}) describing lactate appearance and disappearance, respectively. These two latter terms, common to the three approaches, are important because they represent dynamic dimensions describing the blood lactate kinetics.

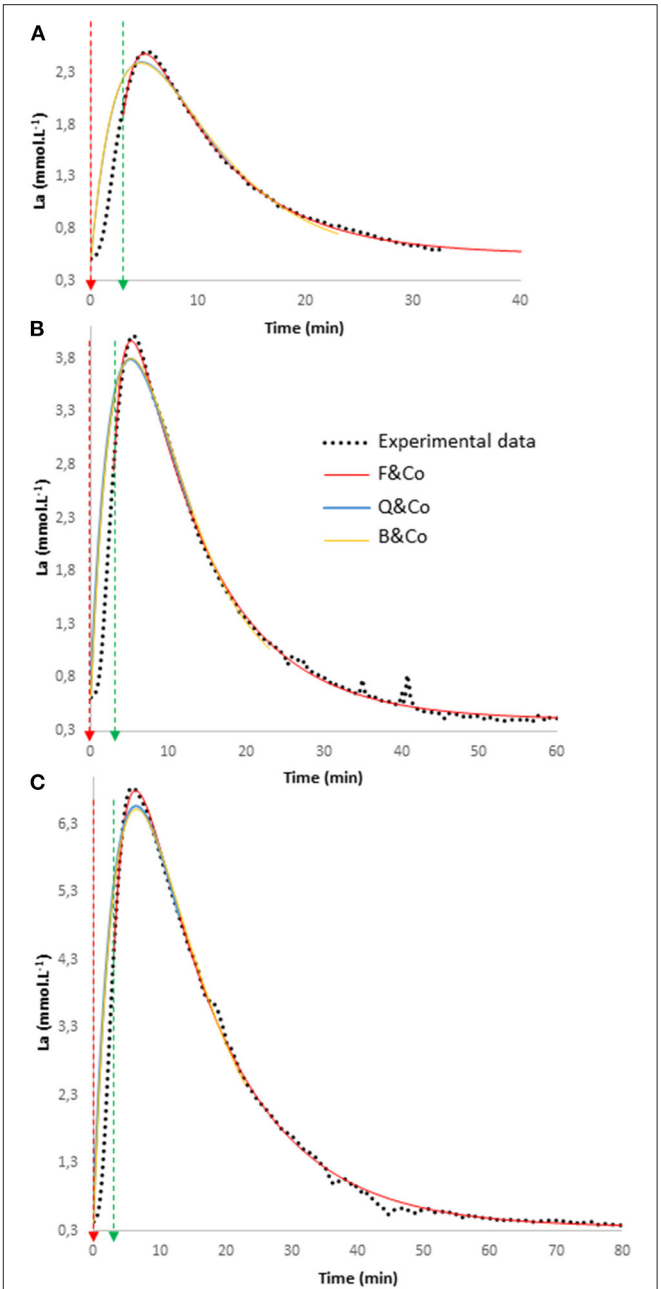

FIGURE 1 | Three experimental blood lactate concentration (La) curves obtained in the same subject during 3-min exercises and the following recoveries at three different exercise intensities [easy **(A)**, moderate **(B)**, and heavy **(C)**, respectively] and the fits from Equations 1–3 applied as Freund and co-workers (F&co), Beneke and co-workers (B&co), and Quittmann and co-workers (Q&co) did (see methods).

BLC_{max} and $TBLC_{max}$ values have been calculated from the parameters of the fits.

During the first series of experiments (Series 1), the three equations were applied over the domain proposed by the respective authors: only during the recovery for F&co, the starting value of physical exercise and the recovery values over 20 or 10 min for B&co or Q&co, respectively. In other words, during this first series, the three equations were applied as performed by Freund et al. (1989), Beneke et al. (2005), and Quittmann et al. (2018).

During the second series of experiments (Series 2), the three equations were fitted to the experimental BLC curves over the entire period of recovery, e.g., the starting value of physical exercise for Equations 2, 3.

During the third series of experiments (Series 3), the equations of F&co were applied to three periods of recovery: to the first 10 min, to the first 20 min, and to the entire period of the recovery.

Statistical Analysis

Values are presented as means and SDs. Statistical analysis was processed using Jasp® software (JASP Team, Version 0.12.2, Amsterdam, the Netherlands). After verification of variable homogeneity and normality by the Shapiro-Wilk test, a sphericity test was performed to compare the means using repeated-measures ANOVA. When the data respected sphericity, Holm's *post-hoc* test was applied. Otherwise, a Greenhouse-Keizer correction factor was applied. If the data did not follow normality, a row ANOVA was performed, with the application of a *post-hoc* Conover test. Correlations between sets of data have been performed. Statistical significance was set at $p < 0.05$.

RESULTS

Series 1

Figure 1 reports three experimental curves of BLCs obtained in the same subject during 3-min exercises and the following

TABLE 1 | Mean values of the velocity constants γ_1 and γ_2 along with the R^2 values of the fits on the experimental data of Freund et al., 1989.

	Mean ± SD			ANOVA (*p* value)	*Post-hoc* (*p* value)			Correlation (*r* value, *p* value)		
	F&co	B&co	Q&co		F&co vs. B&co	F&co vs. Q&co	B&co vs. Q&co	F&co vs. B&co	F&co vs. Q&co	B&co vs. Q&co
n = 38, 3, or 6 min at easy, moderate, or heavy-intensity exercises										
γ_1 (min^{-1})	0.973 ± 0.641	0.320 ± 0.215	0.264 ± 0.131	**<0.001**	**<0.001**	**<0.001**	0.916	0.84, **<0.001**	0.75, **<0.001**	0.87, **<0.001**
γ_2 (min^{-1})	0.0947 ± 0.0289	0.1273 ± 0.0425	0.1475 ±0.0579	**<0.001**	**<0.001**	**<0.001**	0.362	0.71, **<0.001**	0.84, **<0.001**	0.72, **<0.001**
R^2	0.999 ± 0.001	0.993 ± 0.005	0.974 ± 0.021	**<0.001**	**<0.001**	**<0.001**	**<0.001**			
n = 8, 3 min heavy intensity exercises										
γ_1 (min^{-1})	0.721 ± 0.217	0.241 ± 0.080	0.173 ± 0.024	**<0.001**	**<0.001**	**<0.001**	**0.05**	0.86, **0.006**	0.67, 0.064	0.82, **0.013**
γ_2 (min^{-1})	0.0742 ± 0.0155	0.1125 ± 0.0250	0.1517 ± 0.0212	**<0.001**	**0.012**	**<0.001**	0.062	0.26, 0.535	0.69, 0.058	0.30, 0.475
R^2	0.999 ± 0.001	0.988 ± 0.004	0.950 ± 0.016	**<0.001**	0.065	**0.001**	0.065			
n = 7, 6 min heavy intensity exercises										
γ_1 (min^{-1})	0.434 ± 0.298	0.209 ± 0.058	0.261 ± 0.038	**0.017**	**0.012**	0.086	0.306	0.79, 0.035	0.04, 0.929	0.04, 0.928
γ_2 (min^{-1})	0.0634 ± 0.0084	0.0800 ± 0.0168	0.0587 ± 0.0184	**0.003**	**0.004**	0.326	**0.046**	0.78, 0.037	0.33, 0.465	0.22, 0.635
R^2	0.999 ± 0.001	0.993 ± 0.001	0.983 ± 0.005	**<0.001**	**<0.001**	**<0.001**	**<0.001**			

The three equations were applied over the domain proposed by the respective authors, namely, only during the recovery for Freund and co-workers (F&co), the starting value of physical exercise and the recovery values over 20 or 10 min for Beneke and co-workers (B&co) or Quittmann and co-workers (Q&co), respectively. p values ≤ 0.05 are reported in bold.

TABLE 2 | Mean values of maximal blood lactate concentration (BLC_{max}) and time to reach BLC_{max} ($TBLC_{max}$) obtained from the experimental data (ExpData) or predicted by the fits, some of the parameters (γ_1 and γ_2) being reported in **Table 1**.

	ExpData	F&co	B&co	Q&co	F&co vs. ExpData	B&co vs. ExpData	Q&co vs. ExpData
n = 38, 3, or 6 min at easy, moderate, or heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	5.46 ± 2.80	5.42 ± 2.78	5.31 ± 2.71	5.31 ± 2.70	0.072	**<0.001**	**<0.001**
$TBLC_{max}$ (min)	2.06 ± 0.88	1.87 ± 0.84	1.30 ± 1.70	1.53 ± 1.46	0.072	**0.011**	**0.012**
n = 8, 3 min heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	7.20 ± 1.10	7.13 ± 1.14	6.83 ± 1.10	6.89 ± 1.09	0.455	**<0.001**	**0.015**
$TBLC_{max}$ (min)	2.84 ± 0.55	2.80 ± 0.62	3.14 ± 0.79	3.27 ± 0.80	0.831	0.339	0.241
n = 7, 6 min heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	9.48 ± 1.38	9.41 ± 1.38	9.30 ± 1.34	9.25 ± 1.32	0.217	**0.015**	**0.009**
$TBLC_{max}$ (min)	2.39 ± 1.00	2.36 ± 0.71	1.93 ± 1.05	1.67 ± 1.01	0.921	0.465	0.167

p values ≤ 0.05 are reported in bold.

recoveries at three different exercise intensities (easy, moderate, and heavy, respectively), and the model fits from Equations 1–3 following the initial recommendations of F&co, B&co, and Q&co (see methods). Graphically, it can be observed that applying a model during the recovery only (F&co method) describes more closely the recovery experimental values rather than taking into account the pre-exercise value and part of the recovery (B&co or Q&co methods).

Table 1 reports the mean values of the velocity constants (γ_1 and γ_2) along with the R^2 values of the fits on the experimental data. The repeated measures of ANOVAs show significant differences between the three approaches ($p < 0.001$) for all the considered parameters. The *post hoc* tests highlight significant differences between the data obtained by F&co and B&co ($p < 0.001$), and F&co and Q&co ($p < 0.001$). The mean values of γ_1 and γ_2 are not different between the methods applied by B&co and Q&co (**Table 1**). The coefficients of determination R^2 show significant differences ($p < 0.001$) between the three approaches, the R^2 obtained by F&co being the highest. Parameters obtained with the different approaches are on the other hand significantly correlated (**Table 1**). By focusing only on the 3- and 6-min heavy intensity exercises, all parameters are different (**Table 1**), and more importantly, correlations between parameters are not obvious. In that sense, it seems that the longer the exercise, the weaker the correlations (**Table 1**).

Table 2 reports the mean values recorded on the experimental data for BLC_{max} and $TBLC_{max}$ and those obtained from the fits. As it can be seen in **Table 2**, BLC_{max} and $TBLC_{max}$ are underestimated by the approaches of B&co and Q&co.

Series 2

Figure 2 reports three curves obtained in the same subject during 3-min exercises and the following recoveries at three different exercise intensities (easy, moderate, and heavy, respectively) applying the three approaches over the entire period of recovery. Graphically, it can be observed that applying a model during the recovery only (F&co) describes more closely the experimental values than taking into account the pre-exercise value and part of the recovery (B&co or Q&co).

Table 3 reports the mean values of the velocity constants, γ_1 and γ_2, along with the R^2 values of the fits on the experimental data. The repeated measures of ANOVA show significant differences between the three approaches ($p < 0.001$) for all the considered parameters. The *post-hoc* tests highlight significant differences between the data obtained by F&co and B&co ($p < 0.001$), and F&co and Q&co ($p < 0.001$). The mean values of γ_1 and γ_2 are not different between the methods applied by B&co and Q&co (**Table 3**). The coefficients of determination R^2 show significant differences ($p < 0.001$) between the three approaches, the R^2 obtained by the method of F&co being the highest. Parameters obtained with the different approaches are on the other hand significantly correlated (**Table 3**).

Table 4 reports the mean values recorded on the experimental data for BLC_{max} and $TBLC_{max}$ and those obtained from the fits. As it can be seen in **Table 4**, BLC_{max} and $TBLC_{max}$ are underestimated and overestimated, respectively, by the approaches of B&co and Q&co.

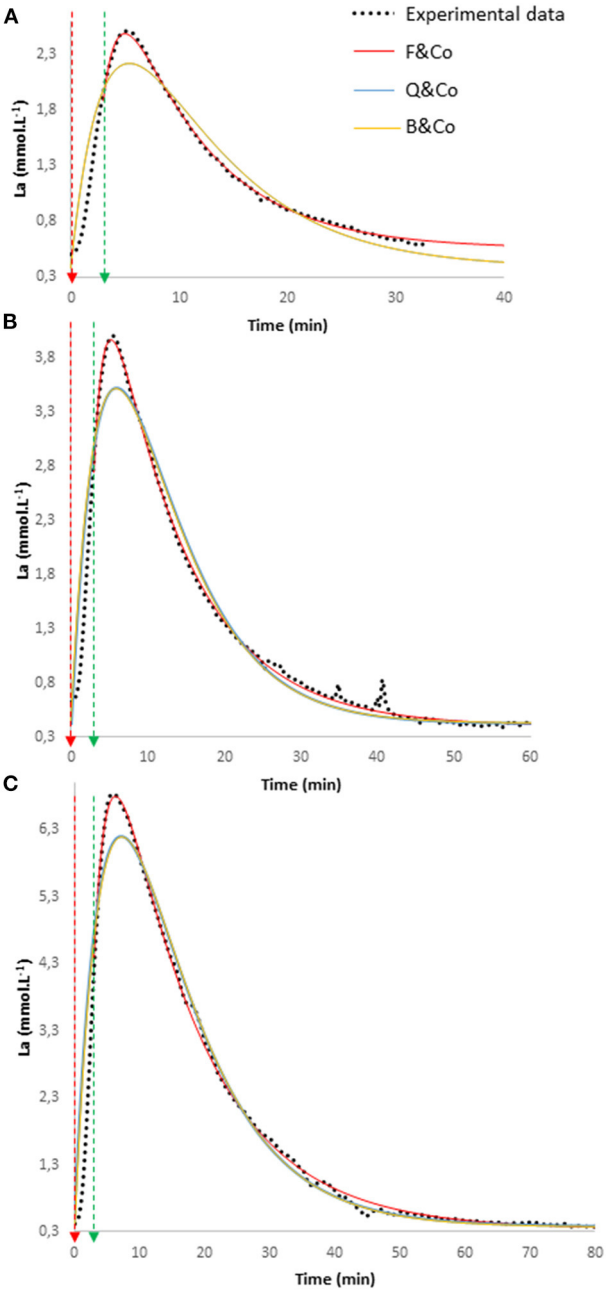

FIGURE 2 | Three experimental blood lactate concentration (La) curves obtained in the same subject during 3-min exercises and the following recoveries at three different exercise intensities [easy **(A)**, moderate **(B)**, and heavy **(C)**, respectively] and the fits from Equations 1–3 applied over the entire period of recovery (for F&co) and taking also into account the initial blood lactate concentration at the beginning of exercise (for B&co and Q&co).

Series 3

Table 5 reports the mean γ_1 and γ_2 values using the model of Freund et al. applied on the entire period of recovery, or only during the first 20 or 10 min of recovery. γ_1 decreases significantly with the shortening of the recovery duration taken

TABLE 3 | Mean values of the velocity constants γ_1 and γ_2 along with the R^2 values of the fits on the experimental data of Freund et al. (1989).

	Mean ± SD			ANOVA (*p* value)	*Post-hoc* (*p* value)			Correlation (*r* value, *p* value)		
	F&co	B&co	Q&co		F&co vs. B&co	F&co vs. Q&co	B&co vs. Q&co	F&co vs. B&co	F&co vs. Q&co	B&co vs. Q&co
n = 41, 3, or 6 min at easy, moderate, or heavy-intensity exercises										
γ_1 (min^{-1})	0.960 ± 0.628	0.210 ± 0.068	0.214 ± 0.066	**<0.001**	**<0.001**	**<0.001**	0.117	0.58, **<0.001**	0.62, **<0.001**	0.99, **<0.001**
γ_2 (min^{-1})	0.0984 ± 0.0403	0.1324 ± 0.0526	0.1280 ± 0.0490	**<0.001**	**<0.001**	**<0.001**	0.08	0.79, **<0.001**	0.81, **<0.001**	0.99, **<0.001**
R^2	0.998 ± 0.003	0.978 ± 0.010	0.978 ± 0.010	**<0.001**	**<0.001**	**<0.001**	0.118			
n = 8, 3 min heavy intensity exercises										
γ_1 (min^{-1})	0.721 ± 0.217	0.222 ± 0.046	0.223 ± 0.044	**0.041**	**0.002**	0.156	**0.05**	0.47, 0.240	0.56, 0.149	0.99, **<0.001**
γ_2 (min^{-1})	0.0742 ± 0.0155	0.0977 ± 0.0349	0.0962 ± 0.0313	**0.009**	**0.001**	**0.002**	0.779	0.99, **<0.001**	0.99, **<0.001**	0.99, **<0.001**
R^2	0.999 ± 0.001	0.984 ± 0.005	0.984 ± 0.005	**<0.001**	**0.003**	**0.003**	1.00			
n = 7, 6 min heavy intensity exercises										
γ_1 (min^{-1})	0.434 ± 0.298	0.138 ± 0.035	0.141 ± 0.032	**0.002**	**0.005**	0.054	0.206	0.81, **0.028**	0.83, **0.022**	0.99, **<0.001**
γ_2 (min^{-1})	0.0634 ± 0.0084	0.0905 ± 0.0218	0.0874 ± 0.0189	**0.002**	**<0.001**	**<0.001**	0.453	0.93, **0.003**	0.95, **0.001**	0.99, **<0.001**
R^2	0.999 ± 0.001	0.989 ± 0.003	0.989 ± 0.003	**<0.001**	**<0.001**	**<0.001**	0.997			

The three equations were applied over the entire period of recovery for Freund and co-workers (F&co) and taking into account the starting value of physical exercise for Beneke and co-workers (B&co) or Quittmann and co-workers (Q&co), respectively. p values ≤ 0.05 are reported in bold.

TABLE 4 | Mean values of maximal blood lactate concentration (BLC_{max}) and time to reach BLC_{max} ($TBLC_{max}$) obtained from the experimental data (ExpData) or predicted by the fits, some of the parameters (γ_1 and γ_2) being reported in **Table 2**.

	ExpData	F&co	B&co	Q&co	F&co vs. ExpData	B&co vs. ExpData	Q&co vs. ExpData
n = 41, 3, or 6 min at easy, moderate, or heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	5.44 ± 2.75	5.40 ± 2.73	4.81 ± 2.48	4.83 ± 2.52	0.13	**<0.001**	**<0.001**
$TBLC_{max}$ (min)	2.08 ± 0.84	1.92 ± 0.82	2.46 ± 1.16	2.45 ± 1.16	0.3	**0.022**	**0.023**
n = 8, 3 min heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	7.20 ± 1.10	7.13 ± 1.14	6.37 ± 1.12	6.37 ± 1.12	0.455	**0.004**	**0.002**
$TBLC_{max}$ (min)	2.84 ± 0.55	2.80 ± 0.62	3.79 ± 0.63	3.78 ± 0.63	1.00	**<0.001**	**<0.001**
n = 7, 6 min heavy-intensity exercises							
BLC_{max} ($mmol.L^{-1}$)	9.48 ± 1.38	9.41 ± 1.38	8.43 ± 1.34	8.63 ± 1.45	0.565	**<0.001**	**<0.001**
$TBLC_{max}$ (min)	2.39 ± 1.10	2.36 ± 0.71	3.09 ± 0.73	3.09 ± 0.73	1.00	0.64	0.064

p values ≤ 0.05 are reported in bold.

into account. On the contrary, γ_2 increases significantly with the shortening of the recovery duration taken into account. Taking into account only the heavy exercises (of 3 or 6 min), the γ_1 and γ_2 values are not different but they are not correlated to each other, meaning that hierarchisation of results/subjects on the basis of their γ_1 and γ_2 values is drastically obscured.

DISCUSSION

The present study aimed to test different models and approaches in depicting and interpreting blood lactate curves during unsteady-state exercise and/or the subsequent recovery. As a whole, it appears that applying models and approaches (i) during exercise and recovery or (ii) during recovery but only over a restricted part of it present pitfalls. In these conditions, the models do not fit adequately to the experimental curves, challenging the interpretation of the velocity constants describing lactate appearance in and disappearance from the blood. Applying models and approaches only during the recovery and over the entire period of recovery (until returning to resting blood lactate values) still appears as the most accurate way to determine the lactate exchange and removal abilities.

Different Approaches and Different Results

Considering the mean values of parameters obtained by Beneke et al. (2005) after application of their model over the exercise and 20 min of recovery in response to a 0.5-min Wingate test, a return to the near resting BLCs after 200 min of recovery can be predicted. This time-to-return for resting blood lactate values is unrealistic. For instance, this duration is much longer (by ∼1 h) than the one (∼140 min) predicted from data obtained by Maciejewski et al. (2013) using the F&co model applied on the entire period of recovery after 3-min all-out exercises, while the exercise was totally exhaustive and led to higher BLCs than in the former case (Beneke et al., 2005). This first comparison

TABLE 5 | Mean values of the velocity constants γ_1 and γ_2 along with the R^2 values of the fits on the experimental data of Freund et al. (1989).

	Mean ± SD			ANOVA (*p* value)	*Post-hoc* (*p* value)			Correlation (*r* value, *p* value)		
	F&co	F&co20'	F&co10'		F&co vs. F&co20'	F&co vs. F&co10'	F&co20' vs. F&co10'	F&co vs. F&co20'	F&co vs. F&co10'	F&co20' vs. F&co10'
n = 40, 3, or 6 min at easy, moderate, or heavy-intensity exercises										
γ_1 (min^{-1})	0.956 ± 0.633	0.724 ± 0.250	0.664 ± 0.380	**<0.001**	**0.001**	**<0.001**	0.504	0.85, **<0.001**	0.64, **<0.001**	0.76, **<0.001**
γ_2 (min^{-1})	0.0966 ± 0.0339	0.1166 ± 0.1057	0.1475 ± 0.0511	**0.007**	**0.021**	**0.003**	0.504	0.80, **<0.001**	0.76, **<0.001**	0.83, **<0.001**
R^2	0.999 ± 0.001	0.998 ± 0.004	0.997 ± 0.001	**<0.001**	**0.007**	**0.016**	**<0.001**			
n = 8, 3 min heavy-intensity exercises										
γ_1 (min^{-1})	0.721 ± 0.217	0.593 ± 0.132	0.591 ± 0.099	0.121	/	/	/	0.619, 0.170	0.052, 0.904	0.245, 0.558
γ_2 (min^{-1})	0.0742 ± 0.0155	0.0836 ± 0.0280	0.0901 ± 0.0862	0.687	/	/	/	0.532, 0.175	**0.753, 0.031**	0.854, **0.007**
R^2	0.999 ± 0.001	0.999 ± 0.001	0.994 ± 0.005	**0.002**	1.00	**0.01**	**0.01**			
n = 7, 6 min heavy-intensity exercises										
γ_1 (min^{-1})	0.473 ± 0.298	0.473 ± 0.347	0.658 ± 0.289	0.156	/	/	/	0.113, 0.810	0.088, 0.851	0.845, **0.017**
γ_2 (min^{-1})	0.0634 ± 0.0084	0.0571 ± 0.0350	0.0467 ± 0.0095	0.156	/	/	/	0.373, 0.411	0.106, 0.822	0.324, 0.478
R^2	0.999 ± 0.001	0.999 ± 0.001	0.993 ± 0.006	**0.018**	0.603	**0.02**	0.054			

The model of Freund and co-workers was applied on the entire period of recovery (F&co), or taking into account only 20 min (F&co20') or 10 min (F&co10') or recovery. p values ≤ 0.05 are reported in bold.

already casts doubt on the fact that the different models and approaches provide congruent results. To answer the question more clearly, our first analysis compared the different parameters obtained after fitting the models on the same experimental set of data (Freund et al., 1989).

In the conditions of applying the equation proposed by each group (i.e., over recovery for F&co, over exercise, and 20 or 10 min of recovery for B&co or Q&co, respectively), the values of the velocity constants are drastically different (**Table 1**). **Figure 1** shows clearly that the equation proposed by F&co fits almost perfectly the recovery curve. On the other hand, the fits from B&co and Q&co equations are more often aside from the experimental data. The deviations are particularly important in the initial phase of the recovery, rendering the determination of the peak BLC during the recovery difficult with the two latter approaches. To illustrate this latter point, BLC_{max} and $TBLC_{max}$ are different from the experimental values using the approaches of B&co and Q&co, but are not different using the approach of F&co (**Table 2**).

One may argue that even if the results are different, correlations still exist between parameters obtained from the different approaches (**Table 1**). However, it is important to keep in mind that blood lactate curves have been obtained in very different conditions (3- or 6-min, easy-, moderate-, or heavy-intensity exercises). Therefore, the question arises as to whether the correlations are still observable for a given exercise, rendering hierarchisation between subjects possible and accurate. **Table 3** reports stratified data according to the performed exercises. As it can be seen, only very few correlations between parameters obtained using the different approaches are obtained.

From that point of view, the lactate exchange and removal abilities (namely, γ_1 and γ_2) obtained from the three approaches are not identical. Two main differences exist between the approaches of F&co, and B&co and Q&co. The two differences lie (i) on the consideration of exercise and recovery or just recovery and (ii) on the considered period of recovery (partial or complete recovery). Therefore, are the differences observed between the approaches related to the first, the second, or a combination of the two differences? The following analyses will try to answer the question.

Effect of Exercise on Blood Lactate Kinetics Parameters (Series 2)

Comparison of data obtained using the three equations on the entire period of recovery (and taking into account the starting exercise value for B&co and Q&co) allows to apprehend the effect of taking into account the exercise on the parameter values. **Table 3** reports the data obtained using this paradigm and underlines very different γ_1 and γ_2 values between the approaches used by F&co and both those used by B&co and Q&co. The fits are also closer to the experimental data using the F&co approach as indicated by the R^2 values and the correspondence with BLC_{max} and $TBLC_{max}$ (**Table 4**; **Figure 2**). Together, these results suggest that considering exercise is detrimental for good fitting and determination of the parameters of the blood lactate recovery kinetics. Actually, this is not so surprising, previous studies have shown that γ_1 and γ_2 values decrease with exercise duration and intensity (Freund et al., 1986, 1989). Consistent with the decrease of γ_1 as the exercise progresses, Juel et al. (1990) observed that during ∼3-min maximal exercises, the net lactate release rate from the active muscle to the blood increased and then tended to plateau, suggesting that lactate exchanges between active muscles and blood are disturbed when maximal exercise progresses. Therefore, if γ_1 and γ_2 are apparently constant during recovery, it appears unrealistic to apply the same constant γ_1 and γ_2 values for exercise and recovery.

Another way to demonstrate the limits of taking into account exercise and recovery is that approaches, such as exercise and recovery, cannot be applied during and after long-lasting (i.e.,

60 min) exercises, while the approach proposed by Freund and co-workers is still valuable (Freund et al., 1990).

Effect of Length of Considered Period During Recovery

We also suspect that the length of the considered period of recovery is critical for an accurate determination of γ_1 and γ_2. A first clue tending to confirm this inference was the lower R^2 values obtained using the approach of Q&co (who considered only the initial 10 min of recovery) compared to the one used by B&co (who considered the first 20 min of recovery). To further investigate the importance of the length of the considered recovery period, we performed two additional analyses. First, a comparison was performed between the data obtained in Series 1 (considering only 20 or 10 min of recovery) and Series 2 (considering the entire period of recovery) for 3- and 6-min heavy-intensity exercises using the approaches of B&co and Q&co (**Tables 1**, **3**). According to the period of recovery taken into account, values of parameters changed. Second, a comparison was performed between the data obtained after exercise using the F&co approach applied (i) on the full length, (ii) over 20 min, or (iii) over 10 min of recovery (Series 3; **Table 5**). Clearly, γ_1 and γ_2 values are different according to the length of recovery taken into account (**Table 5**). The shorter the recovery, the lower the γ_1 and the higher the γ_2. These complementary analyses indicate that it is better to take into account the entire recovery period. Actually, as it can be seen in Figures for moderate or heavy exercise, there are important inflexion points on the curves beyond the 20 min of recovery. These points need to be taken into account for precise γ_2 determinations.

Limitations

In the present report, we criticised the fact that taking into account exercise and recovery are not very precise in so far as the dynamical parameters that drive the blood lactate curve (according to the models depicted here), namely, the lactate exchange and removal abilities, change during exercise. In that regard, applying constant values during exercise and recovery makes little sense. Actually, the same criticism could be made to the F&co model and approach. Indeed, the probability that γ_1 and γ_2 remain constant over the entire period of recovery is very low. Indeed, if it is obvious that at the onset of the recovery, γ_1 and γ_2 reflect the physiological state of the subject at that time, it is also obvious that at the end of the recovery, γ_1 and γ_2 are almost returned to resting conditions. Nevertheless, the fits of the model are very close to the experimental data ($R^2 > 0.999$) so that everything appears as if the lactate exchange and removal abilities remain constant all over the recovery.

Perspectives

Although theoretically not perfect, the model and approach proposed by F&co seem for the moment the most accurate to describe the blood lactate curve during recovery and determine accurately two important aspects of its kinetics: the lactate exchange and removal abilities (namely, γ_1 and γ_2, respectively). Therefore, we recommend using this model and its domain of application for the entire period of recovery.

In the present study, the models and approaches proposed by B&co and Q&co have been applied on a set of data obtained during and after 3- or 6-min exercises. In such circumstances, strong limitations of these models and approaches to describe the blood lactate curve and thus to accurately determine γ_1 and γ_2 appeared. However, we acknowledge that the impact of exercise on the fits and on the determination of γ_1 and γ_2 would have been much lower if very short exercises (typically 15 or 30 s) had been used. Therefore, we strongly encourage the future scholars who would like to use the B&co or Q&co models and approaches to strictly apply these models (i) only during and after very short exercises and importantly (ii) on the entire period of recovery. Indeed, whatever the model and approach are, a precise determination of the lactate removal ability (γ_2) requires a long period of recovery.

CONCLUSION

Taken together, caution should be taken concerning the interpretation of the velocity constants obtained from the models and approaches proposed by Beneke et al. (2005) and Quittmann et al. (2018) if exercise is longer than 30 s and if the recovery is incomplete. At present, it seems that the model and approach proposed by F&co, although tedious due to the long period of recovery studied, remains the most accurate way to describe the blood lactate recovery curve and determine the lactate exchange and removal abilities during recovery.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licences/restrictions: Data have been previously published. Original data have been obtained from the authors. Requests to access these datasets should be directed to Hubert Freund, hubert.freund@wanadoo.fr.

ETHICS STATEMENT

We reused data already published. At the time of the experiments, the study complied with the law of the country for studies involving human participants and with the declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM and HF designed and conducted this study and wrote the first draft of the article. RD, MG, MC, and DB analyzed data. All authors critically reviewed the draft and approved the final version for publication.

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