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Short Communication

Lactate dehydrogenase supports lactate oxidation in mitochondria isolated from different mouse tissues

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A B S T R A C T

Research over the past seventy years has established that mitochondrial-L-lactate dehydrogenase (m-L-LDH) is vital for mitochondrial bioenergetics. However, in recent report, Fulghum et al. concluded that lactate is a poor fuel for mitochondrial respiration [1]. In the present study, we have followed up on these findings and conducted an independent investigation to determine if lactate can support mitochondrial bioenergetics. We demonstrate herein that lactate can fuel the bioenergetics of heart, muscle, and liver mitochondria. Lactate was just as effective as pyruvate at stimulating mitochondrial coupling efficiency. Inclusion of LDH (sodium oxamate or GSK 2837808A) and pyruvate dehydrogenase (PDH; CPI-613) inhibitors abolished respiration in mitochondria energized with lactate. Lactate also fueled mitochondrial ROS generation and was just as effective as pyruvate at stimulating H_2O_2 production. Additionally, lactate-induced ROS production was inhibited by both LDH and PDH inhibitors. Enzyme activity measurements conducted on permeabilized mitochondria revealed that LDH is localized in mitochondria. In aggregate, we can conclude that mitochondrial LDH fuels bioenergetics in several tissues by oxidizing lactate.

1. Introduction

The presence of m-L-LDH in mitochondria was first proposed in 1951 and confirmed twenty years later [2,3]. Since then m-L-LDH has been found to be integral in supplying energy for neural function and normal muscle physiology and fueling the bioenergetics of various tissues and cell types [4–9]. The reduction of pyruvate to lactate in the cytoplasm and its subsequent re-oxidation to pyruvate in the matrix of mitochondria has also been suggested to serve as an alternative NADH shuttle for the transfer of hydrides to complex I [10]. Uptake of lactate by mitochondria is facilitated by the monocarboxylate transporter (MCT), which is coupled to the return of a proton to the matrix. The MCT isozyme solute anion carrier-16A1 (SLC16A1) has been found to facilitate lactate import in several cell and tissue types [11,12]. The existence of m-L-LDH in mitochondria has also been confirmed in plants and yeast (reviewed in Ref. [5]).

The importance of m-L-LDH in mitochondrial bioenergetics is underscored by its inclusion in the MitoCarta [13]. However, despite the overwhelming evidence supporting the existence of m-L-LDH, its presence in mitochondria is not universally accepted. Indeed, several studies have reported that mitochondria do not oxidize lactate due to the absence of an LDH isozyme [1,14–16]. It was also hypothesized that mitochondria cannot oxidize lactate due to a high NADH/NAD^+ ratio, which would prevent its conversion to pyruvate [15]. However, we find this cannot be the case given that 1) other fuel combusting pathways that converge on the Krebs cycle also produce NADH (e.g. fatty acid oxidation) and 2) this hypothesis ignores the high turnover rate of NADH in the matrix due to complex I activity.

The enthusiastic debate surrounding the existence of m-L-LDH led to the publication of an article titled "*The Mitochondrial L-Lactate Dehydrogenase Affair*" in 2014 [13]. To counter skepticism concerning the existence of m-L-LDH, the authors of this article provided a road map to properly test lactate-fueled mitochondrial bioenergetics [13]. This includes emphasis on using appropriate buffer systems, the importance of using coupled mitochondria to fully interrogate the states of respiration, and the use of an inhibitor tool kit coupled with various assays to measure lactate oxidation [13]. However, in a recent report published in Redox Biology, Fulghum et al. concluded that lactate cannot fuel the bioenergetics of striated muscle due to the absence of m-L-LDH [1]. These findings directly contradict previous studies showing that lactate can fuel mitochondria in striated muscle and various other tissues as well as a myriad of cell types [9,17–23]. Using the guide supplied by Passarella et al., we counter the conclusions drawn by Fulghum et al. and demonstrate that mitochondria oxidize lactate [13].

2. Experimental

Chemicals: Pyruvate, malate, lactate, sodium oxamate, 3-methyl-2-

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oxo valeric acid, malonate, superoxide dismutase, horseradish peroxidase, oligomycin, antimycin A, ADP, $MgCl_2$, KH_2PO_4, delipidated bovine serum albumin, mannitol, sucrose, NAD^+, HEPES, EGTA, and KCl were purchased from Sigma-Aldrich. CPI-613 and GSK 2837808A were purchased from Santa Cruz. Amplex UltraRed reagent was purchased from Thermo Fisher.

Animals and Tissue isolation: Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care . C57BL/6 N mice were purchased from Charles River at 9 weeks of age, housed at 25 °C on a 12-h day/night light cycle, and provided water and chow *ad libitum* (Teklad Global 18% Protein Rodent Diet). At 10 weeks of age, mice were placed under heavy anesthesia (5% isoflurane) and euthanized by cervical dislocation. Liver and heart tissue were ablated and placed in ice-cold mitochondrial isolation buffer containing mannitol (220 mM), EGTA (1 mM), sucrose (70 mM), and HEPES (10 mM) (MESH; pH 7.4) containing 0.5% fatty acid free BSA (MESH-B). Skeletal muscle tissue dissected from the fore and hind limbs and pectoral region was pooled and stored in ice-cold basic medium (BM; KCl (140 mM), HEPES (20 mM), EGTA (1 mM) and $MgCl_2$ (5 mM) at pH 7.0).

Isolation of mitochondria: All steps were carried on ice or at 4 °C. Briefly, excised livers were cut into small pieces, rinsed thoroughly, and then minced on a Teflon watch glass. Minced tissue was then homogenized by hand in MESH-B. The isolation of liver mitochondria was carried out as described in Ref. [24]. The mitochondrial pellet was resuspended in ~500 μL MESH giving a final protein concentration of ~16–18 mg/mL. Collection of heart mitochondria was conducted as described in Ref. [24]. Dissected hearts were first cut into small pieces and washed thrice in MESH-B. The heart pieces were minced and homogenized by hand in MESH-B containing 1 U of subtilisin A. The homogenate was centrifuged at 800 × *g* for 9 min. The supernatant was collected and centrifuged at 10,000 × *g* for 9 min. The mitochondrial pellet was then washed and resuspended in 100 μL of MESH, giving a final concentration of protein equivalents to mitochondria of ~5 mg/mL. Muscles were pooled and placed in basic medium (BM; KCl; 140 mM, HEPES; 20 mM, EGTA; 1 mM, and $MgCl_2$; 5 mM, pH 7.0 with 6 N HCl). Skeletal muscle was cleaned, weighed, and minced on a Teflon watch glass. Minced tissue was then placed in 15 mL of homogenization medium (BM containing ATP; 1 mM, 1% (w/v) BSA and 1 U subtilisin A). Samples were homogenized using a variable speed reversible homogenizer (Glas-Col). Muscle mitochondria were isolated as described in Ref. [25]. The mitochondrial pellet was resuspended in ~200 μL of BM to give a final protein concentration equivalent to mitochondria of ~10 mg/mL. Protein concentration was determined with the Bradford Assay.

Measurements of H_2O_2 production: Mitochondria were allowed to equilibrate in buffer for 10 min at 25 °C in reaction buffer containing oligomycin (4 μg/mL) to induce state 4 respiratory conditions. Mitochondria were also incubate for 10 min at 25 °C with a mixture of the following ROS production inhibitors; CPI-613 (250 μM; α-keto acid dehydrogenase inhibitor), 3-methyl-2-oxo valeric acid (KMV; 10 mM; α-ketoglutarate dehydrogenase inhibitor), and malonate (10 mM; complex II inhibitor) to ensure changes resorufin fluorescence was associated with mitochondrial H_2O_2 production and not due to the spontaneous oxidation of the AUR. Reaction mixtures also contained either CPI-613 (250 μM) to inhibit pyruvate dehydrogenase or the LDH inhibitors sodium oxamate (50 mM) and GSK 2837808A (GSK; 75 μM). Of note, oxamate is a weak LDH inhibitor that has been found to impede the activities of other enzymes [26]. However, it has been documented that oxamate does not inhibit pyruvate dehydrogenase [27]. Reactions were initiated with the addition of pyruvate (10 mM) or lactate (10 mM) with malate (2 mM) and changes in fluorescence were tracked as described previously using Synergy Mx monochromatic multi-well plate reader using Gen5 software (Biotek) [24].

Polarographic measurement of mitochondrial bioenergetics: The rate of O_2 consumption under the different states of respiration was measured using an Oxytherm Clark-type electrode system (Hansatech). Liver, cardiac, and skeletal muscle mitochondria were then diluted in the reaction chamber containing respiration buffer (MESH-B with KH_2PO_4; 10 mM and $MgCl_2$; 2 mM). LDH and PDH inhibitors CPI-613 (250 μM), sodium oxamate (50 μM), and GSK (75 μM) were also added in certain cases. Mitochondria were then allowed to equilibrate for a few minutes to achieve a stable baseline for O_2 consumption. States 2–4 respiration were then measured as described in Ref. [24] using pyruvate (10 mM) or lactate (10 mM) with malate (2 mM) as substrates. The respiratory control ratio (RCR) was calculated as described in Ref. [24].

Lactate dehydrogenase activity assay: Matrix and intermembrane space lactate dehydrogenase activities using permeabilized mitochondria as described in Ref. [24]. Reaction mixtures were supplemented with NAD^+ (0.5 mM) and lactate (1 mM) and the production of NADH was tracked at A_{340} for 5 min using a Synergy Mx monochromatic multi-well plate reader using Gen5 software (Biotek). LDH inhibitors oxamate (50 mM), or GSK (75 μM) were included as a control. Results were corrected for background absorbance.

Data Analysis: Graph pad prism 6 software was utilized for all statistical analyses. All data is represented as the mean ± standard error of mean (SEM) with N = 4. AUR, Oxytherm, and LDH activity assays were performed 4 times and in duplicate. One and two-way analysis of variance (ANOVA) with a Tukey's post hoc test was employed for all experimental results except in Fig. 1B where a paired two-tailed Student's T-Test was used. Statistical significance was represented as follows: *; $P \leq 0.05$, **; $P \leq 0.01$, ***; $P \leq 0.001$, ****; $P \leq 0.0001$.

3. Results and discussion

3.1. Lactate supports respiration

Analyses of the different states of respiration revealed that lactate was just as effective as pyruvate at driving the consumption of O_2 by heart, muscle, and liver mitochondria (Fig. 1). Induction of state 3 respiration by the inclusion of ADP induced a robust increase in O_2 consumption by heart mitochondria oxidizing pyruvate or lactate (Fig. 1A). A similar observation was made with muscle mitochondria except state 3 respiration was significantly higher when pyruvate served as the substrate (Fig. 1A). However, lactate induced a robust increase in ADP-stimulated respiration in isolated muscle mitochondria, nonetheless. Furthermore, lactate was just as effective as pyruvate at supporting state 3 respiration by liver mitochondria (Fig. 1A). Inhibition of ATP synthase with oligomycin (state 4 respiration) decreased the rate of respiration by heart, muscle and liver mitochondria. The rate of nonphosphorylating respiration was similar in cardiac, muscle, and liver mitochondria oxidizing either pyruvate or lactate (Fig. 1A). Next, we calculated the respiratory control ratio for mitochondria oxidizing pyruvate or lactate, a proxy measure for mitochondrial coupling. Fig. 1B shows that heart mitochondria were more coupled due to a significantly higher RCR ratio when lactate served as the substrate. Additionally, RCR ratios for muscle and liver mitochondria oxidizing pyruvate or lactate were not significantly different (Fig. 1B). However, since the RCR were ~5–8, we can conclude that mitochondria prepared from muscles or liver were well-coupled regardless of which substrate was serving as a fuel (Fig. 1B). Intriguingly, two previous studies demonstrated that lactate was actually a better fuel for mitochondrial respiration than pyruvate [7,9]. Here, we observed that lactate was as effective as pyruvate at stimulating respiration. This discrepancy could be attributed to the loss of LDH bound to the outer membrane. A previous study did find that LDH localizes to the outer mitochondrial membrane [28]. Therefore, it is possible that the discrepancy between our findings and these two previous studies is due to the loss of externally bound LDH during the preparation of our mitochondria.

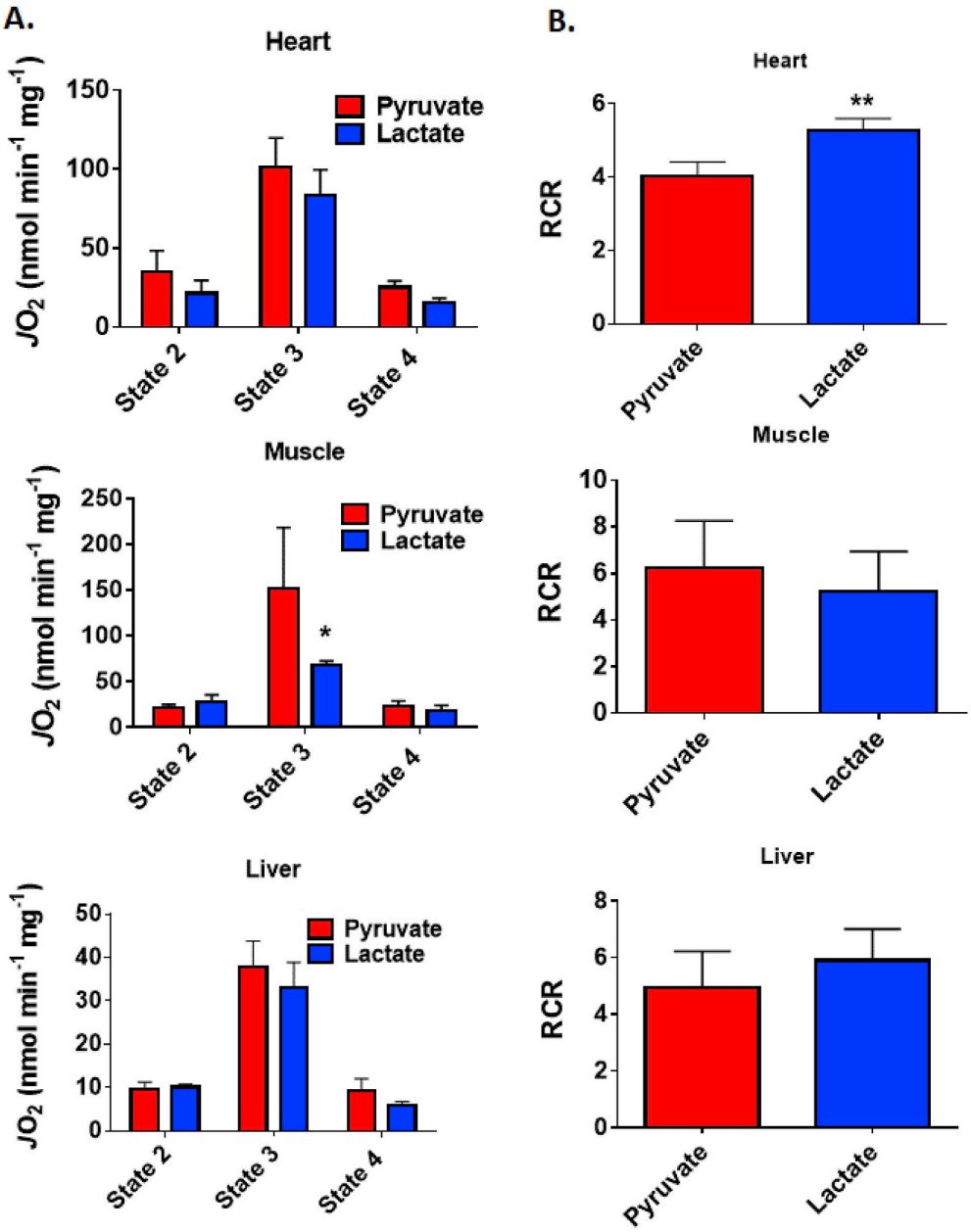

Fig. 1. Lactate is just as effective as pyruvate at supporting mitochondrial respiration. **A.** State 2–4 respiration were measured using mitochondria isolated from cardiac, muscle, and liver tissue. State 2 respiration was stimulated by the addition of pyruvate or lactate (10 mM) and malate (2 mM) followed by the addition of ADP to induce state 3 conditions. State 4 respiration was then measured by the addition of oligomycin (4 μg/mL). N = 4, mean ± SEM, 2-way ANOVA with a Tukey's post-hoc test. **B.** Respiratory control ratios (RCR) were calculated using the state 3 and 4 respiration rate values collected in **A**. N = 4, mean ± SEM, paired two-tailed Student's T-Test.

3.2. Lactate induces H_2O_2 production

Both pyruvate and lactate were equally effective at inducing a measurable rate of H_2O_2 production in heart and muscle mitochondria (Fig. 2). Lactate was slightly more effective at inducing ROS production by heart mitochondria (Fig. 2). Inclusion of the inhibitor cocktail (CPI-613 + KMV + malonate) almost completely abolished H_2O_2 production confirming that the oxidation of pyruvate or lactate was responsible for inducing mitochondrial ROS formation (Fig. 2). Lactate induced a significantly higher rate of H_2O_2 production (~2-fold increase) in liver mitochondria when compared to samples treated with pyruvate (Fig. 2). One possible explanation for this effect could be related to NADH turnover. NADH accumulation increases H_2O_2 production by PDH through 1) reverse electron transfer to the E3 subunit or 2) feedback inhibition [29,30]. Liver mitochondria display low rates of NADH turnover when compared to heart and muscle, which is reflected by the differences in O_2 consumption by these mitochondria in Fig. 1A. This could potentially increase ROS production by PDH since the oxidation of 1 mol of lactate by mitochondria yields 5 mol of NADH when compared to pyruvate, which yields 4 mol of NADH.

3.3. Lactate-supported respiration depends on LDH

GSK and oxamate alone or in combination almost abolished O_2 consumption by heart and muscle mitochondria energized with lactate (Fig. 3). Furthermore, GSK and oxamate almost abolished ADP-dependent respiration in mitochondria from heart and muscle (Fig. 3). GSK and oxamate also inhibited O_2 consumption in liver mitochondria fueled with lactate (Fig. 3). However, these inhibitors were not as effective at limiting lactate oxidation when compared to the rates of respiration in heart and muscle mitochondria. We also conducted experiments using reaction mixtures containing CPI-613, an inhibitor for α-keto acid dehydrogenases like pyruvate dehydrogenase, to determine if it can also abolish lactate-supported respiration. Inclusion of CPI-613 almost abolished lactate-stimulated O_2 consumption by heart and muscle mitochondria (Fig. 3). Additionally, CPI-613 was more effective at limiting mitochondrial lactate oxidation, which is attributed to the fact that it is a potent inhibitor for α-keto acid dehydrogenases and thus

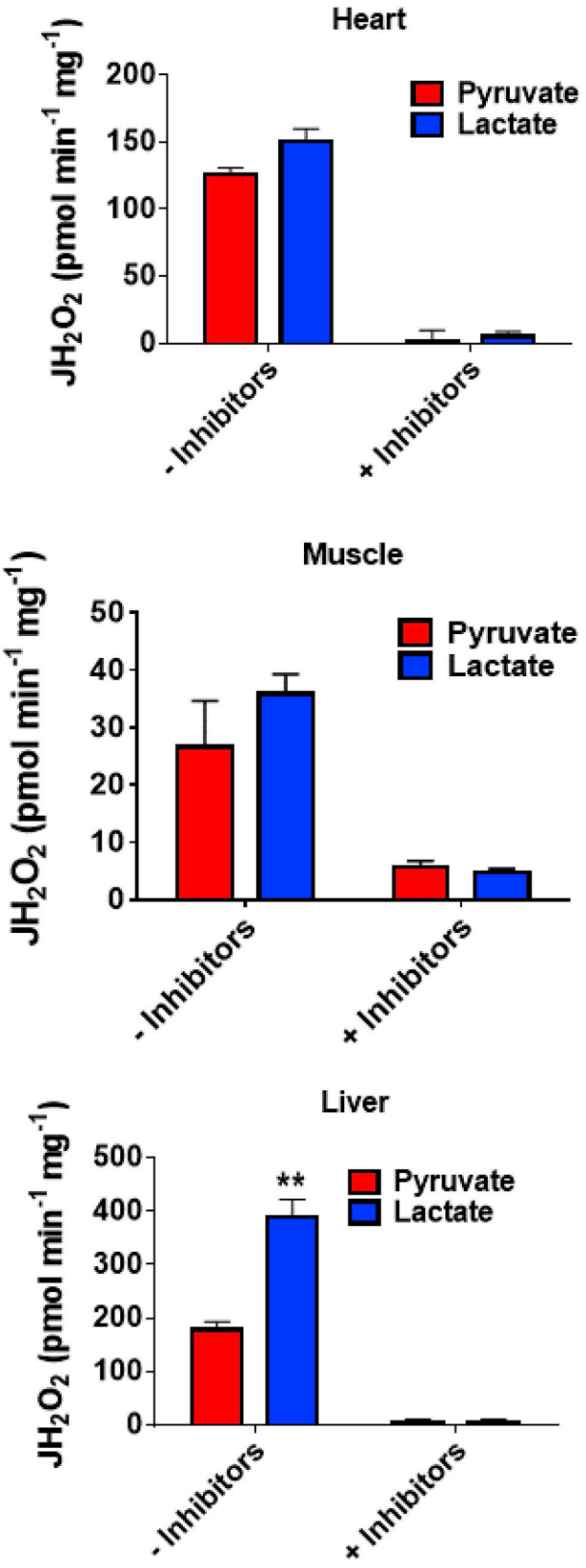

Fig. 2. Lactate is a substrate for mitochondrial H_2O_2 production. Mitochondria from cardiac, muscle and liver tissue were supplemented with pyruvate or lactate (10 mM) and malate (2 mM) and then ROS production was tracked using the Amplex UltraRed assay. An inhibitor cocktail consisting of CPI-613, 3-methyl-2-oxo valeric acid, and malonate served as the control. N = 4, mean ± SEM, 2-way ANOVA with a Tukey's post-hoc test.

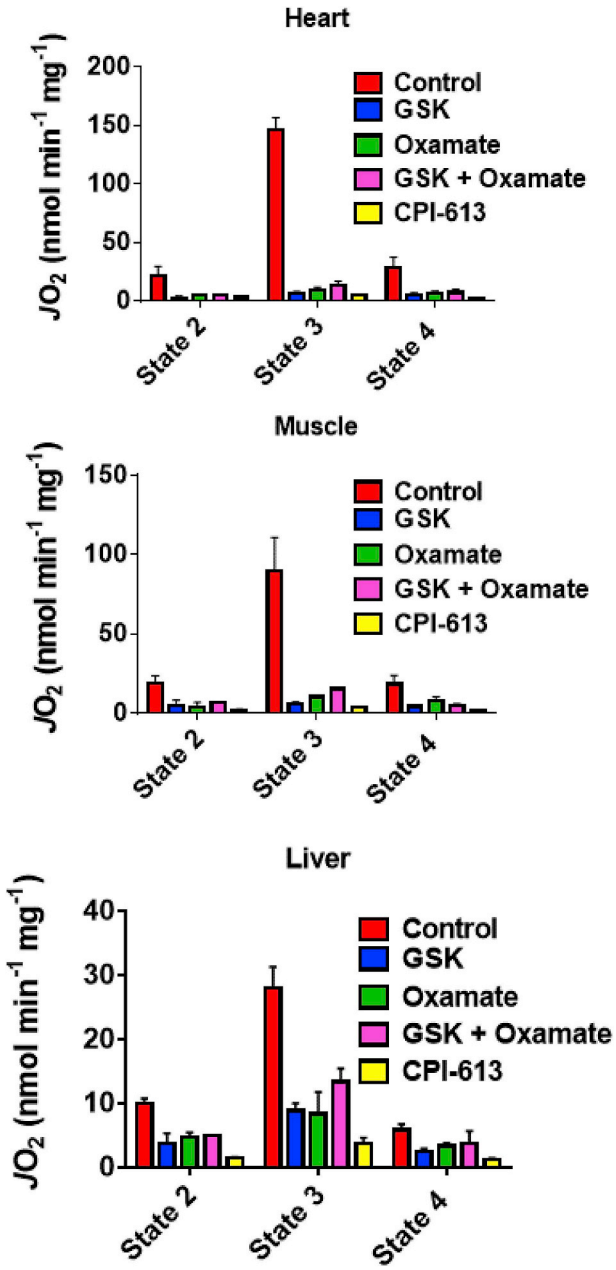

Fig. 3. Mitochondrial lactate oxidation relies on its conversion to pyruvate by m-L-LDH. Cardiac, muscle, and liver mitochondria were treated with or without sodium oxamate, GSK, CPI-613, or a combination of sodium oxamate + GSK and then energized with lactate (10 mM) and malate (2 mM). The impact of the inhibition of m-L-LDH and PDH on lactate-stimulated respiration was then measured. N = 4, mean ± SEM, 2-way ANOVA with a Tukey's post-hoc test.

diminishes flux through the Krebs cycle. CPI-613 was also an effective inhibitor for O_2 consumption by liver mitochondria energized with lactate. This demonstrates that use of lactate for respiration first requires its conversion to pyruvate by m-L-LDH.

Inclusion of GSK, oxamate, or CPI-613 inhibited H_2O_2 production by mitochondria from all three tissues energized with lactate (Fig. 4). In heart and muscle mitochondria, oxamate and GSK induced a several fold decrease in H_2O_2 production (Fig. 4). A similar effect was observed with CPI-613. Furthermore, inclusion of both oxamate and GSK in reaction mixtures almost completely abolished H_2O_2 production by heart and muscle mitochondria (Fig. 4). Finally, treating liver mitochondria with oxamate, GSK, a combination of both oxamate + GSK, or CPI-613 almost completely abolished ROS production (Fig. 4).

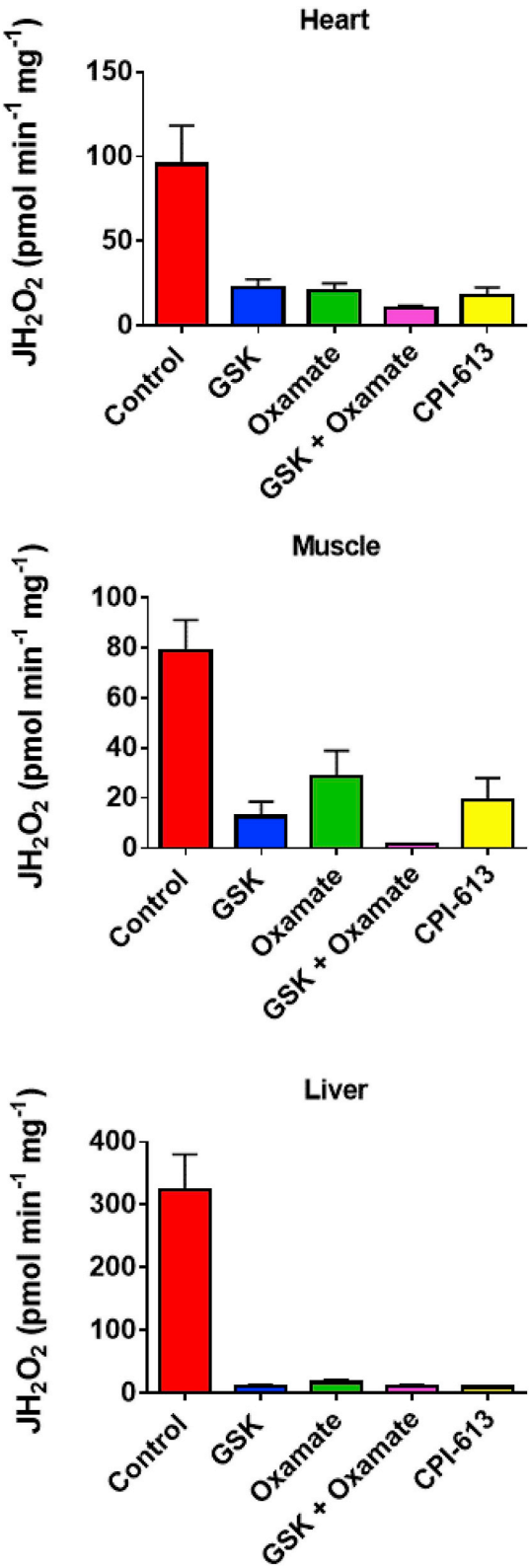

Fig. 4. Oxidation of lactate by m-L-LDH induces mitochondrial H_2O_2 production. Cardiac, muscle, and liver mitochondria were treated with or without sodium oxamate, GSK, CPI-613, or a combination of sodium oxamate + GSK and then energized with lactate (10 mM) and malate (2 mM). The impact of the inhibition of m-L-LDH and PDH on lactate-stimulated H_2O_2 production was then measured. N = 4, mean ± SEM, 1-way ANOVA with a Tukey's post-hoc test.

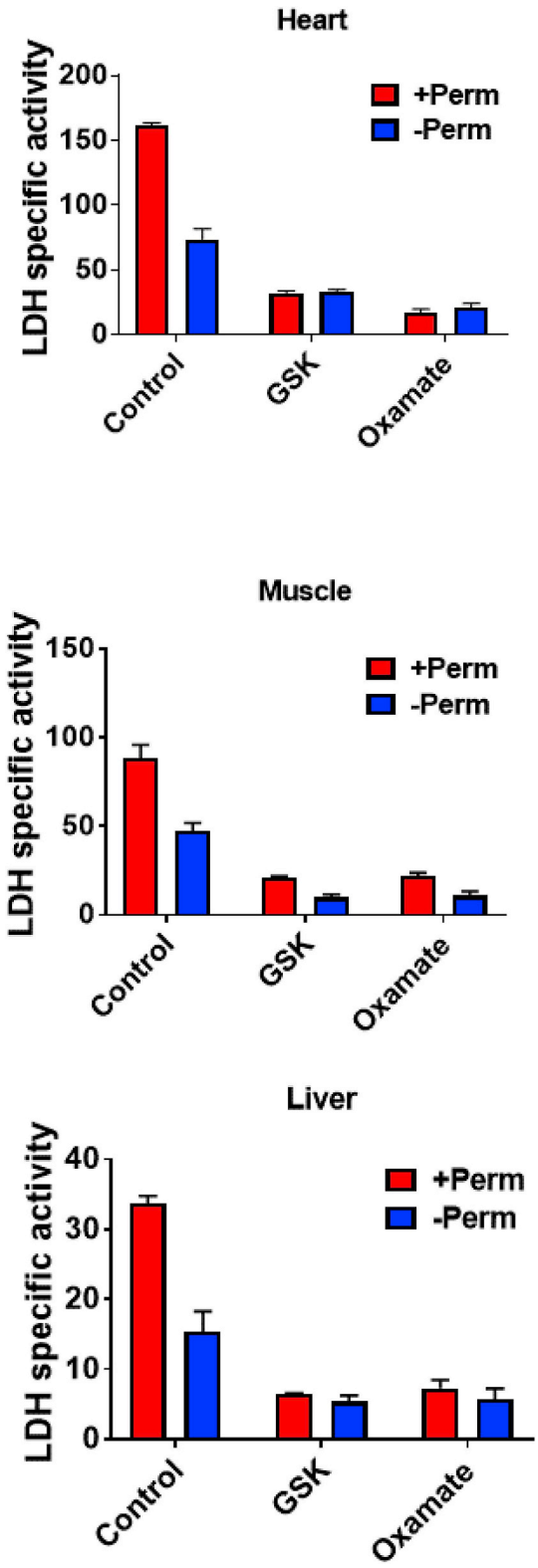

Fig. 5. Mitochondria contain LDH. Mitochondria from striated muscle and liver tissue were treated with or without Triton X-100 (membrane permeabilizer) and then LDH activity was measured. Sodium oxamate and GSK were included as controls. N = 4, mean ± SEM, 2-way ANOVA with a Tukey's post-hoc test.

3.4. Mitochondria harbor LDH activity

Measurement of the rate of NADH production in permeabilized mitochondria (+Perm) oxidizing lactate revealed that samples

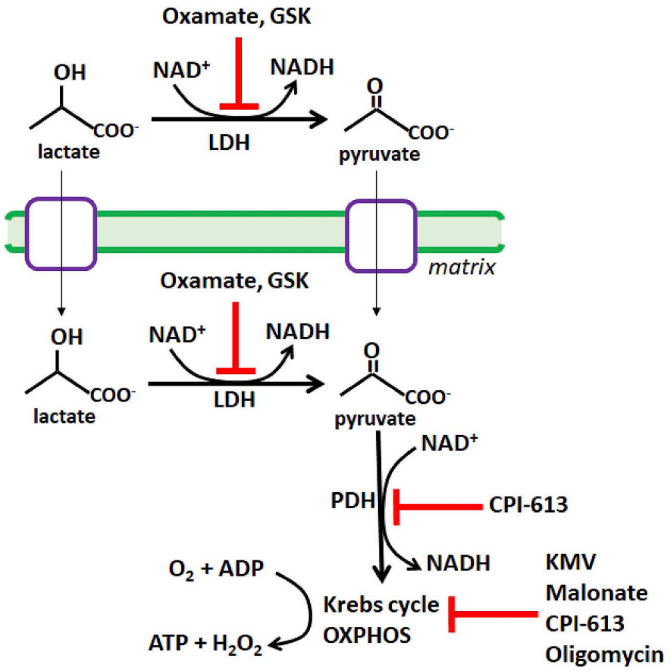

Fig. 6. Summary of findings and sites of inhibition for the different chemicals used in this study.

enriched from heart, muscle, and liver tissue all retained m-L-LDH activity (Fig. 5). Additionally, this activity could be inhibited with oxamate and GSK (Fig. 5). We also observed that mitochondria not subjected to Triton X-100 treatment (non-permeabilized; -Perm) retained some LDH activity, suggesting that it is also localized either to the intermembrane space or tethered to the outer mitochondrial membrane (Fig. 5). The area external to the matrix environment harbored ~50% LDH activity, which was also inhibited by adding either oxamate or GSK to the reaction chambers (Fig. 5). This could be attributed to cytoplasmic contamination, even though mitochondria were washed before assays. It is also worth pointing out that cytosolic LDH has been shown to localize to the outer mitochondrial membrane [28]. It has also been reported that LDH localizes to the intermembrane space. However, LDH activity did increase by ~2-fold in permeabilized mitochondria from heart, muscle and liver tissue, respectively, indicating an isozyme is housed in the matrix. Furthermore, it has been demonstrated that LDH localizes to the intermembrane space and matrix [9].

Fifty years of research has established that lactate can support respiration and cell metabolism [4]. The utilization of lactate as a fuel *in vivo* has been shown to rely on a lactate shuttling system; once formed by one cell, lactate can be taken up by neighboring cells to support mitochondrial bioenergetics or other reactions (*e.g.* gluconeogenesis) [31–33]. The mitochondria-associated LDH isoform has been shown to oxidize lactate and fuel the bioenergetics of various cell types and protect from oxidative distress [10] [22,23,34,35]. Adipocytes have also recently been found to use a unique lactate shuttling system where it serves as an autocrine messenger for the regulation of lipolysis by serving as a ligand for orphan G-protein coupled receptros [36]. Brooks et al. also recently published a comprehensive review on the topic detailing these functions as well as some other intriguing roles lactate fulfills in the human body, including participating in a hypothetical microbe-to-enterocyte lactate shuttle in the gut and host-pathogen interactions [4].

Using approaches suggested by Passarella et al. [13], we confirmed that mitochondria from striated muscles and liver tissue oxidize lactate to fuel respiration and ROS production, findings that contradict the published report by Fulghum et al. [1]. Inhibitor studies revealed that lactate needs to be converted to pyruvate before it can be oxidized further (Fig. 6). Conversion of lactate to pyruvate also fuels H_2O_2 production. Finally, activity assays demonstrated that the matrix and space external to the mitochondrial inner membrane harbor LDH activities (Fig. 6). Collectively, we can conclude lactate is a good fuel for mitochondrial bioenergetics in mammalian cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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