L-Lactate transport into rat heart mitochondria and reconstruction of the L-lactate/pyruvate shuttle

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In vitro reconstruction of the L-lactate/pyruvate shuttle has been performed, which allows NADH oxidation outside rat heart mitochondria. Such a shuttle occurs due to the combined action of the novel mitochondrial L-lactate/pyruvate antiporter, which differs from the monocarboxylate carrier, and the mitochondrial L-lactate dehydrogenase. The rate of L-lactate/pyruvate antiport

proved to regulate the shuttle *in vitro*.

Key words: lactate mitochondrial transport, lactate mitochondrial uptake, lactate/pyruvate antiporter, reducing-equivalent transfer system.

INTRODUCTION

Although L-lactate production by working muscle is usually considered to be the result of a lack of muscle oxygenation, it also occurs in contracting skeletal muscle under fully aerobic conditions, and L-lactate is always present in blood, muscle, heart and other tissues [1]. Moreover, arterial L-lactate concentration is low in mammals during sustained exercise because production is balanced by gluconeogenesis and, more importantly, by mitochondrial oxidation [2,3]. In particular, cardiac metabolism becomes more dependent on carbohydrates during congestive heart failure and L-lactate may be used as an important respiratory substrate [4]. This raises the question as to how mitochondria participate in L-lactate oxidation and whether oxidation of L-lactate can contribute to the oxidation of cytosolic NADH. Indeed, the existence of mitochondrial L-lactate dehydrogenase (L-LDH), already reported in rat heart mitochondria (RHM) [5], has been confirmed, with the enzyme localized in both the intermembrane space and matrix fraction [6]. However, whether and how L-lactate can enter mitochondria remains to be established. In this study, we show the occurrence of the novel L-lactate/pyruvate antiporter which, together with mitochondrial L-LDH, allows NADH oxidation outside mitochondria via the L-lactate/pyruvate shuttle.

EXPERIMENTAL

Materials and isolation of RHM

All reagents and enzymes were from Sigma (St. Louis, MO, U.S.A.). Mitochondrial substrates were used as Tris salts at pH 7–7.3.

RHM were isolated as described in [7] from male Wistar rats (200–250 g) fed *ad libitum*. RHM were suspended in a medium containing 70 mM sucrose, 220 mM mannitol, 5 mM Hepes/Tris, pH 7.4, and 0.1 mM EDTA in the presence of 0.5 mg/ml BSA. Mitochondrial protein was determined according to [8]. RHM showed a respiratory control index of 3 ± 0.14 with 5 mM

succinate used as respiratory substrate in the presence or absence of 0.5 mM ADP, as measured in five experiments.

Fluorimetric and photometric assays

Changes in the redox state of mitochondrial pyridine nucleotides were monitored fluorimetrically, using a PerkinElmer LS-5 luminometer with excitation and emission wavelengths of 334 and 456 nm, respectively [9,10]. The NAD(P)H fluorescence was calibrated as in [9].

Pyruvate uptake was monitored following the reduction of intra-mitochondrial NAD(P)⁺, caused by externally adding the substrate to mitochondria that had been incubated previously with 1.25 μ M FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). Rotenone (2 μ g) was added 3–5 min later to prevent oxidation of the newly synthesized NAD(P)H via the respiratory chain.

Pyruvate appearance outside mitochondria was monitored by using the pyruvate-detecting system [P.D.S.; consisting of 200 μ M NADH plus L-LDH, 1 enzymic unit (e.u.)] [9] and then following photometrically the NADH oxidation caused by externally added L-lactate, which itself proved to have no effect on the enzymic reactions. Absorbance was measured at 334 nm. Since the equilibrium constant of L-LDH is very high (3.6×10^4 M⁻¹; $K_{eq} = [\text{lactate}][\text{NAD}^+]/[\text{pyruvate}][\text{NADH}]$ at pH 7), we checked whether the presence of L-lactate could make the P.D.S. reaction outside mitochondria rate-limiting with respect to the transport rate. Pyruvate (20 μ M) was completely reduced to L-lactate in the presence of externally added L-lactate (15 mM), with a rate that was one order of magnitude higher than that of pyruvate efflux from RHM with 15 mM L-lactate added.

The ϵ_{334} value measured for both NADH and NADPH under our experimental conditions was found to be 6.3 mM⁻¹·cm⁻¹.

Control experiments were carried out to confirm that all of the compounds used in this study did not affect the enzymes used to reveal metabolite appearance outside mitochondria.

In each experiment, the intactness of the mitochondrial inner membrane was confirmed by checking the rate of NADH oxidation in the absence of any substrate. When the rate was

Abbreviations used: α -CCN⁻, α -cyano-4-hydroxycinnamate; L-LDH, L-lactate dehydrogenase; P.D.S., pyruvate-detecting system; RHM, rat heart mitochondria; e.u., enzymic unit.

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higher than 5% of that measured following metabolite efflux, mitochondria were not used. In each experiment NADH was added to both the sample and reference cuvettes, and the rate of NADH oxidation was measured as the difference between the rates measured in the two cuvettes, thus allowing us to obtain the correct rate of absorbance change resulting from the reactions outside mitochondria [9].

The rates of both fluorescence and absorbance change were obtained as tangents to the initial stages of the progress curve and expressed as either nmol of NAD⁺ reduced/mg of protein or nmol of NADH oxidized/mg of mitochondrial protein.

RESULTS

Pyruvate appearance outside RHM due to externally added L-lactate

To gain some insight into the L-lactate permeability of mitochondria, RHM were incubated in the presence of NADH (0.2 mM) and L-LDH (1 e.u.), and NADH absorbance was monitored as a function of time. In the presence of added L-LDH, no significant change in the absorbance was found, showing that RHM were intact and that the pyruvate concentration in the extra-mitochondrial phase was negligible. As a result of the addition of 3 mM L-lactate to RHM, a decrease in the absorbance was found, indicating the appearance of pyruvate outside RHM (Figure 1A). A possible explanation for this finding is that L-lactate can enter RHM, perhaps in exchange for endogenous pyruvate; inside mitochondria, due to the NAD-dependent L-LDH [6], pyruvate is formed which, in turn, can exit the mitochondria in exchange for further L-lactate in a carrier-mediated process. Externally added rotenone (2 μ g) was found consistently to completely prevent the appearance of pyruvate outside RHM, and phenylsuccinate, an inhibitor of many translocators that cannot enter mitochondria [11], strongly inhibited pyruvate efflux. Interestingly, 10 μ M α -cyano-4-hydroxycinnamate (α -CCN⁻), a potent inhibitor of the pyruvate carrier [12], had no effect on the pyruvate appearance outside mitochondria. In the same experiment, pyruvate uptake by RHM was investigated in the absence and presence of α -CCN⁻ (Figure 1B). The addition of pyruvate (0.5 mM) to RHM pre-incubated with rotenone (2 μ g) caused a rapid increase in the fluorescence of the intra-mitochondrial pyridine nucleotides, thus showing pyruvate's capability of entering mitochondria, where it is oxidized to acetyl CoA with reduction of intra-mitochondrial NAD(P)⁺. α -CCN⁻ (10 μ M) was found to completely inhibit the rate of fluorescence increase (Figure 1B). Use was made of Triton X-100 (0.5%; see Figure 1B inset) to ascertain whether the rate of pyruvate uptake via its own carrier is the limiting step in the measured fluorescence increase: more than twice the increase in NAD⁺ reduction rate was seen after Triton X-100 addition to RHM (compared with the rate measured before Triton X-100 addition), showing that the rate of fluorescence increase reflects the pyruvate uptake into RHM.

In order to check whether matrix pyruvate could efflux from mitochondria itself, RHM were incubated in the presence of NADH (0.2 mM) plus rotenone (2 μ g) and L-LDH (0.3 e.u.), and then alanine (1 mM) and 2-oxoglutarate (0.5 mM) were added (Figure 1C), thus providing the substrate pair for mitochondrial alanine aminotransferase [13]. No change in the NADH absorbance was observed, illustrating the lack of pyruvate outside the mitochondria. As a result of L-lactate addition, which cannot cause pyruvate efflux in the absence of alanine and 2-oxoglutarate, a decrease in absorbance was found which showed the efflux of intra-mitochondrial pyruvate. Such an efflux proved to be prevented completely by amino-oxyacetate, a penetrant

Figure 1 Lactate transport into RHM

(A) Pyruvate appearance outside RHM due to externally added L-lactate. RHM (0.75 mg of protein) were incubated at 25 °C in 2 ml of the standard medium consisting of 200 mM sucrose, 10 mM KCl, 1 mM MgCl₂ and 20 mM Hepes/Tris, pH 7.2, in the presence of the P.D.S. Where indicated, 2 μ g of rotenone (ROT), 4 mM phenylsuccinate (PHESUC) or 10 μ M α -CCN⁻ were added. At the second arrow, 3 mM L-lactate was supplemented. Numbers along traces indicate the initial rates of pyruvate appearance (V_0), expressed as nmol of NADH oxidized/min per mg of protein. (B) Pyruvate (PYR) uptake by RHM. RHM (0.5 mg of protein) were incubated at 25 °C in the presence of 2 ml of standard medium plus 2 μ g of rotenone. At the arrow, pyruvate (0.5 mM) was added in either the absence or presence of 10 μ M α -CCN⁻ (added 1 min before the pyruvate). The rate of fluorescence increase is expressed as nmol of intra-mitochondrial NAD⁺ reduced/min per mg of mitochondrial protein. In the inset, Triton X-100 (0.5%; TX-100) was added to RHM previously supplemented with rotenone (2 μ g), NAD⁺ (0.2 mM) and pyruvate (0.5 mM) and the change of fluorescence monitored continuously. (C) Pyruvate appearance outside pyruvate-loaded RHM due to externally added L-lactate. RHM (0.5 mg of protein) were incubated at 25 °C in 2 ml of standard medium in the presence of the P.D.S. plus 2 μ g of rotenone. As indicated, 1 mM alanine (ALA), 0.5 mM 2-oxoglutarate (2-OG) and 3 mM L-lactate were added. Either 10 μ M α -CCN⁻ or 1 mM amino-oxyacetate (AOA) was added to RHM 1 min before L-lactate ($\pm$ α -CCN⁻ indicates that the experimental traces obtained in the absence and presence of α -CCN⁻ had the same rate). The rate of pyruvate appearance in the extra-mitochondrial phase due to L-lactate addition was expressed as nmol of NADH oxidized/min per mg of protein.

inhibitor of mitochondrial transaminases [13], but totally unaffected by α -CCN⁻ (Figure 1C). In a parallel experiment (results not shown), carrier-free [¹⁴C]pyruvate was added to mitochondria following preincubation with alanine and 2-oxoglutarate; after a further 2 min 1 mM pyruvate was added. A 90% reduction in mitochondrial radioactivity was found, whereas no significant

Figure 2 Phenylsuccinate inhibition of the pyruvate appearance outside RHM due to externally added L-lactate

RHM (0.5 mg of protein) were incubated at 25 °C in 2 ml of standard medium (see Figure 1A) in the presence of the P.D.S. The rate of the pyruvate appearance outside RHM due to externally added L-lactate was measured, as in Figure 1(A), by using 3 (○) or 15 (●) mM L-lactate in either the absence or presence of increasing phenylsuccinate concentrations. Results are expressed as nmol of NADH oxidized/min per mg of protein.

change was found due to pyruvate addition in the presence of $10 \mu\text{M } \alpha\text{-CCN}^-$.

Phenylsuccinate inhibition of the pyruvate appearance outside RHM due to externally added L-lactate

In order to gain some insight into the mechanism by which L-lactate causes pyruvate efflux from RHM, the rate of pyruvate appearance was measured at two L-lactate concentrations (3 and 15 mM) in the absence and presence of increasing phenylsuccinate concentrations. Phenylsuccinate was found to inhibit the appearance of pyruvate outside RHM in a non-competitive manner ($K_i \approx 3 \text{ mM}$; Figure 2). Interestingly, the y intercepts of the lines fitting the experimental points obtained in the presence of inhibitor coincide with the experimental points calculated in the absence of inhibitor. According to the control strength criterion [14], this demonstrates that the rate of pyruvate appearance outside RHM is a measure of the transport process rate.

Dependence on L-lactate concentration and certain features of the L-lactate/pyruvate antiport

The dependence of the L-lactate/pyruvate antiport rate as a function of increasing L-lactate concentration was investigated. In agreement with the occurrence of carrier-mediated transport, saturation kinetics were found, with $V_{\max}$ and K_m values equal to $25 \pm 0.45 \text{ nmol/min per mg of protein}$ and $3.6 \pm 0.18 \text{ mM}$, respectively (Figure 3). In the same experiment, the $V_{\max}$ and K_m values of pyruvate uptake into RHM were $13.4 \pm 0.76 \text{ nmol/min per mg of protein}$ and $0.3 \pm 0.03 \text{ mM}$, respectively (results not shown).

The pH profile of the pyruvate efflux rate due to externally added L-lactate (15 mM) was also investigated in the range of

Figure 3 Dependence of the rate of L-lactate/pyruvate antiport on L-lactate concentration

RHM (0.5 mg of protein) were incubated at 25 °C in 2 ml of standard medium (see Figure 1A) in the presence of the P.D.S. The rate of L-lactate/pyruvate antiport was measured as a function of increasing L-lactate concentration and expressed as nmol of NADH oxidized/min per mg of protein.

Figure 4 pH profile of L-lactate/pyruvate antiport

RHM (0.5 mg of protein) were incubated at 25 °C in the presence of the P.D.S. The rate of L-lactate/pyruvate antiport was measured at the indicated pH values by using 15 mM L-lactate, and is expressed as nmol of NADH oxidized/min per mg of protein.

pH 5–8. The maximum rate was measured at pH 5, with a bell-shaped profile in the range of pH 6–8 (Figure 4). Control strength experiments were carried out at different pH values as in

Figure 2: the transport was found to limit the rate of absorbance decrease at the investigated pH (results not shown).

The capability of certain inhibitors of mitochondrial carriers to affect the L-lactate/pyruvate antiporter was investigated. α -CCN⁻ (10–500 μ M), 1,2,3-benzenetricarboxylate (1 mM) and carboxyatractyloside (5 μ M), which are potent inhibitors of pyruvate, tricarboxylate and ADP/ATP carriers, respectively [11], failed to inhibit the rate of L-lactate/pyruvate antiport. Conversely, 0.1 mM bathophenanthroline, a metal-complexing agent that inhibits several mitochondrial carriers [15], was found to inhibit the rate of L-lactate/pyruvate antiport by $\approx$ 50 % of the control.

DISCUSSION

In this paper we show the *in vitro* reconstruction of the novel L-lactate/pyruvate shuttle in RHM due to the combined action of the L-lactate/pyruvate antiporter and mitochondrial L-LDH. The criteria usually accepted for assessing the existence of a carrier-mediated process, i.e. the occurrence of saturation kinetics and inhibition by non-penetrant compounds, have been applied successfully. Consistently, the application of the control strength criterion, using phenylsuccinate, allowed us to rule out the possibilities that pyruvate can be formed in the external mitochondrial compartments at a significant rate and that pyruvate can efflux by free diffusion and/or via the monocarboxylate carrier.

The $V_{\max}$ value for the L-lactate/pyruvate antiport (25 nmol/min per mg of protein) has the same order of magnitude as other mitochondrial carriers [11]; the relatively high K_m value ($\approx$ 3.5 mM) for L-lactate accounts for the capability of RHM to remove excess L-lactate with high efficiency. Consistently, the highest cytosolic L-lactate was suggested to be about 10 mM [6]. This novel carrier can be distinguished from the pyruvate, tricarboxylate and ADP/ATP translocators by virtue of its insensitivity to their specific inhibitors, α -CCN⁻, 1,2,3-benzenetricarboxylate and carboxyatractyloside, respectively. Moreover, the non-competitive nature of phenylsuccinate inhibition of the L-lactate/pyruvate antiporter excludes the possibility that the L-lactate/pyruvate antiport can be mediated by both dicarboxylate and oxidocarboxylate carriers [11]. Finally, the evidence of inhibition due to bathophenanthroline suggests the presence of metal ion(s) in this carrier and shows that L-lactate transport does not occur via the bathophenanthroline-insensitive phosphate carrier (results not shown, and [15]).

We propose that the cytosolic NADH can be partially oxidized via the L-lactate/pyruvate shuttle reconstructed here. Such a shuttle could work together with the malate/aspartate and α -glycerophosphate shuttles [16] and the malate/oxaloacetate shuttle, recently shown in rat heart left ventricle mitochondria (A. Atlante, T. Seccia, A. Pirrelli, E. Marra and S. Passarella, unpublished work). The lactate/pyruvate shuttle is regulated by the transport step and is assumed to be active essentially at a very low pH, perhaps determined by a high L-lactate concentration in the mitochondrial microenvironment.

The participation of mitochondria in balancing production and oxidation of L-lactate 'as part of an intracellular lactate

shuttle' has been proposed recently [6,17–19]. These authors suggest that both L-lactate and pyruvate can enter RHM via the monocarboxylate carrier (MCT1), sensitive to α -CCN⁻. On the contrary, we show here that the novel L-lactate/pyruvate antiporter proved to be insensitive to α -CCN⁻.

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REFERENCES

- Connett, R. J., Honing, C. R., Gayeski, T. E. and Brooks, G. A. (1990) Defining hypoxia: a systems view of VO_2 , glycolysis, energetics, and intracellular PO_2 . *J. Appl. Physiol.* **68**, 833–842.
- Brooks, G. A., Wolfel, E. E., Groves, B. M., Bender, P. R., Butterfield, G. E., Cymerman, A., Mazzeo, R. S., Sutton, J. R., Wolfe, R. R. and Reeves, J. T. (1992) Muscle accounts for glucose disposal but not blood lactate appearance during exercise after acclimatization to 4,300 m. *J. Appl. Physiol.* **72**, 2435–2445.
- Huie, M. J., Casazza, G. A., Horning, M. A. and Brooks, G. A. (1996) Smoking increases conversion of lactate to glucose during submaximal exercise. *J. Appl. Physiol.* **80**, 1554–1559.
- Johannsson, E., Lunde, P. K., Heddle, C., Sjaastad, I., Thomas, M. J., Bergersen, L., Halestrap, A. P., Blackstad, T. W., Ottersen, O. P. and Sejersted, O. M. (2001) Upregulation of the cardiac monocarboxylate transporter MCT1 in a rat model of congestive heart failure. *Circulation* **104**, 729–734.
- Brandt, R. B., Laux, J. E., Spainhour, S. E. and Kline, E. S. (1987) Lactate dehydrogenase in rat mitochondria. *Arch. Biochem. Biophys.* **259**, 412–422.
- Brooks, G. A., Dubouchaud, H., Brown, M., Sicurello, J. P. and Butz, C. E. (1999) Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1129–1134.
- Valenti, D., Barile, M. and Passarella, S. (2000) AZT inhibition of the ADP/ATP antiport in isolated rat heart mitochondria. *Int. J. Mol. Med.* **6**, 93–96.
- Waddell, W. J. and Hill, C. A. (1956) A simple ultraviolet spectrophotometric method for determination of protein. *J. Lab. Clin. Med.* **48**, 311–314.
- Passarella, S., Atlante, A. and Quagliariello, E. (1985) Oxaloacetate permeation in rat kidney mitochondria: pyruvate/oxaloacetate and malate/oxaloacetate translocators. *Biochem. Biophys. Res. Commun.* **129**, 1–10.
- Atlante, A., Passarella, S., Pierro, P. and Quagliariello, E. (1994) Proline transport in rat kidney mitochondria. *Arch. Biochem. Biophys.* **309**, 139–148.
- LaNoue, F. K. and Schoolwerth, A. C. (1984) Metabolic transport in mammalian mitochondria. In *Bioenergetics* (Ernster, L., ed.), pp. 221–261, Elsevier, Amsterdam.
- Halestrap, A. P. (1975) The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* **148**, 85–96.
- Marra, E., Passarella, S., Casamassima, E., Perlino, E., Doonan, S. and Quagliariello, E. (1985) Kinetic studies of the uptake of aspartate aminotransferase and malate dehydrogenase into mitochondria *in vitro*. *Biochem. J.* **228**, 493–503.
- Valenti, D., Barile, M., Quagliariello, E. and Passarella, S. (1999) Inhibition of nucleoside diphosphate kinase in rat liver mitochondria by added 3'-azido-3'-deoxythymidine. *FEBS Lett.* **444**, 291–295.
- Passarella, S., Palmieri, F. and Quagliariello, E. (1973) The role of metal ions in the transport of substrates in mitochondria. *FEBS Lett.* **38**, 91–95.
- Rupert, B. E., Segar, J. L., Schutte, B. C. and Scholz, T. D. (2000) Metabolic adaptation of the hypertrophied heart: role of the malate/aspartate and alpha-glycerophosphate shuttles. *J. Mol. Cell Cardiol.* **32**, 2287–2297.
- Brooks, G. A., Brown, M. A., Butz, C. E., Sicurello, J. P. and Dubouchaud, H. (1999) Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1. *J. Appl. Physiol.* **87**, 1713–1718.
- Brooks, G. A. (2000) Intra- and extra-cellular lactate shuttles. *Med. Sci. Sports Exerc.* **32**, 790–799.
- Dubouchaud, H., Butterfield, G. E., Wolfel, E. E., Bergman, B. C. and Brooks, G. A. (2001) Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **278**, E571–E579.