ENERGY-DEPENDENT REGULATION OF THE STEADY-STATE CONCENTRATIONS OF THE COMPONENTS OF THE LACTATE DEHYDROGENASE REACTION IN LIVER

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1. Introduction

It has been known for some years that the components of the lactate dehydrogenase reaction in liver are maintained in a steady-state believed to be close to thermodynamic equilibrium [1,2]. The ratio, [lactate]/[pyruvate] has thus been considered to reflect the 'redox state' (the ratio of 'free' [NAD]/[NADH]) within the cytoplasmic compartment of the hepatic cell [3]. Analogous investigations of mitochondrial dehydrogenase systems in liver have led to the conclusion that the mitochondrial redox state is ~100-times more reduced than that of the cytoplasm [3]. It has been pointed out that because of these differences in redox state, the transfer of reducing-equivalents from cytoplasmic to mitochondrial NAD(H) pools would be against the electrochemical potential gradient and therefore likely to be energy-dependent [4,5]. However, few relevant experimental observations using whole cell preparations have been described. The work presented here provides evidence for a direct involvement of energy in the maintenance of the steady-state [lactate]/[pyruvate] ratio in isolated liver cells.

2. Materials and methods

Isolated liver cells from Hooded Wistar rats (250–280 g body wt), starved for 24 h to deplete liver glycogen, were prepared by a modification [6] of the method in [7]. For one series of experiments, cells were obtained from rats treated with clofibrate, (ethyl-2-(4-chlorophenoxy)-2-methylproprionate), by daily subcutaneous injection (100 mg/kg body wt) for 14–21 days. These cells were used because of their resistance to damage during incubation with rotenone. For all experiments, the cells (80–120 mg wet wt) were incubated in 2 ml of a balanced bicarbonate–saline medium [8] with a gas phase of 95% O_2, 5% CO_2 at 37°C for 40 min. Albumin, 2.5% w/v (final conc.) was included in the incubation medium when palmitate was an added substrate.

Respiration was measured in the presence of CO_2 by a manometric method [9]. Metabolites were measured by standard enzymic techniques, as in [8,10], on neutralized perchloric acid extracts of the incubated cells. The quantity of [1-^{14}C]palmitate completely oxidized in the tricarboxylic acid cycle was estimated by collecting $^{14}CO_2$ in phenethylamine, after acidification of the vessel contents. Enzymes for metabolite determination were from Boehringer-Mannheim (Sydney, NSW), collagenase was a product of Worthington Biochemical Corp. (Freehold, NJ). [1-^{14}C]Palmitate was from the Radiochemical Centre (Amersham, UK). Rotenone was obtained from Sigma (St Louis, MO).

3. Results

3.1. *Restoration of the steady-state [lactate]/[pyruvate] ratio following a lactate load*

When cells were incubated with lactate, added at 2.5–15 mM, the initially high [lactate]/[pyruvate] ratio was rapidly decreased, both by removal of lactate for gluconeogenesis and by accumulation of pyruvate. The formation of glucose from lactate (0.65 ± 0.05 μmol . min^{-1} . g^{-1} (mean ± SE, $n = 10$)) requires no net transfer of reducing equivalents from cytoplasm to mitochondria. On the other hand, in the absence of alternative cytoplasmic hydrogen sinks,

Table 1
Effects of DNP on lactate metabolism and adenine nucleotide concentrations

DNP added (μM)	Oxygen uptake	Lactate removal	Pyruvate accumulation	Glucose accumulation	ATP	ADP
	(μmol . min^{-1} . g wet wt^{-1})				(μmol . g wet wt^{-1})	
0	3.64	1.92	0.37	0.59	1.78	0.46
25	5.52	2.06	0.33	0.43	1.61	0.69
50	6.34	2.45	0.24	0.41	1.51	0.82
75	7.53	2.74	0.20	0.39	1.37	1.10
100	5.70	2.68	0.17	0.28	1.08	1.33

Isolated liver cells from starved normal rats were incubated for 40 min with 10 mM lactate and the concentrations of DNP indicated. Values are from a representative experiment

pyruvate accumulation necessarily implies transfer of lactate hydrogen to O_2 by way of the mitochondrial respiratory chain. For cells exposed to 10 mM lactate, the rate of pyruvate accumulation gradually declined from an initial value of 1.26 ± 0.08 μmol . min^{-1} . g^{-1} (n = 13) to zero after ~40 min, by which time the [lactate]/[pyruvate] ratio was at a steady-state value of 8.04 ± 0.32 (n = 20).

3.2. *Energy dependence of hepatic [lactate]/[pyruvate] ratio*

To examine whether or not the maintenance of the [lactate]/[pyruvate] ratio was energy-dependent, cells were incubated with 2,4-dinitrophenol (DNP). This uncoupling agent affected both the rate at which the [lactate]/[pyruvate] ratio returned to steady-state after a lactate load, and the value of the steady-state ratio achieved (table 1, fig.1). Respiration was stimulated by DNP, the maximum rate being observed at 75 μM. At this concentration, rates of glucose synthesis were depressed only ~33%, despite a 3-fold fall in [ATP]/[ADP]. Evidently, the enhanced O_2-uptake allowed sufficient ATP formation to meet the demands of gluconeogenesis, even though oxidative phosphorylation was partially uncoupled. Balance studies (table 1) show that in the absence of DNP >80% of the lactate removed could be accounted for as glucose and pyruvate. As the DNP concentration was raised, an increasing proportion of the lactate was not accounted for by these products and can be assumed to have been completely oxidized in the tricarboxylic acid cycle.

The set of curves in fig.1 illustrate the rate of approach to steady-state of the [lactate]/[pyruvate] ratio under control conditions and in the presence of various concentrations of DNP. The most obvious effect of DNP is the rise in the final steady-state value of the ratio with each incremental increase in DNP concentration. However, other striking relationships are demonstrated in fig.2. It is apparent that the rate

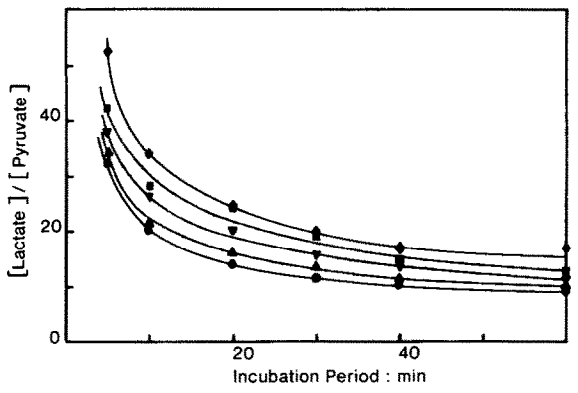

Fig.1. Effect of DNP on restoration of the steady-state ratio of [lactate]/[pyruvate] by isolated liver cells.

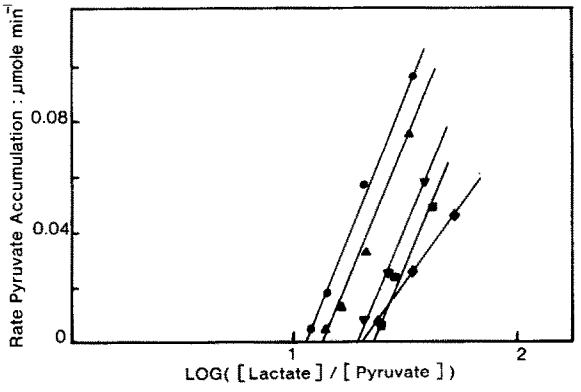

Fig.2. Effect of DNP on the relationship between rate of pyruvate accumulation and logarithm of [lactate]/[pyruvate]. These figures were derived from the data of the experiment described in table 1: (–●–) No DNP; (–▲–) 25 μM DNP; (–▼–) 50 μM DNP; (–■–) 75 μM DNP; (–◆–) 100 μM DNP.

Table 2
Effects of rotenone and palmitate on the [lactate]/[pyruvate] ratio

Additions	Roten-one	O_2-uptake	$^{14}CO_2$	Acetoacetate	3-Hydroxy-butyrate	Lactate	Glucose	Pyruvate (μmol)	*L/P*
		($\mu mol \cdot min^{-1} \cdot g\ wet\ wt^{-1}$)							
Lactate	–	3.78 ± 0.09				−1.77 ± 0.15	0.59 ± 0.04	1.35 ± 0.04	8.2
Palmitate	–	4.15 ± 0.18	0.140 ± 0.020	0.89 ± 0.06	1.47 ± 0.10				
Lactate, palmitate	–	4.93 ± 0.19	0.480 ± 0.060	0.84 ± 0.06	0.64 ± 0.08	−2.40 ± 0.14	0.65 ± 0.04	0.66 ± 0.07	10.4
Lactate	+	1.15 ± 0.28				−0.23 ± 0.08	0.03 ± 0.01	0.41 ± 0.07	49.9
Palmitate	+	1.50 ± 0.07	0.072 ± 0.012	0.05 ± 0.01	0.70 ± 0.06				
Lactate, palmitate	+	1.40 ± 0.11	0.044 ± 0.008	0.06 ± 0.03	0.98 ± 0.08	−0.50 ± 0.13	0.04 ± 0.01	0.25 ± 0.01	78.2
Acetoacetate	+	1.11 ± 0.15		−0.83 ± 0.11	1.40 ± 0.19				
Lactate, acetoacetate	+	0.99 ± 0.20		−1.33 ± 0.17	2.66 ± 0.30	−0.92 ± 0.10	0.06 ± 0.02	0.07 ± 0.01	255
Palmitate, acetoacetate	+	3.01 ± 0.16	0.160 ± 0.020	−1.54 ± 0.09	3.88 ± 0.21				
Lactate, palmitate acetoacetate	+	3.15 ± 0.13	0.320 ± 0.029	−1.96 ± 0.21	4.10 ± 0.22	−0.59 ± 0.09	0.16 ± 0.03	0.62 ± 0.04	25.6
Lactate, palmitate, acetoacetate, DNP	+	2.85 ± 0.21	0.123 ± 0.005	−1.87 ± 0.17	4.12 ± 0.19	−0.42 ± 0.06	0.04 ± 0.01	0.08 ± 0.01	125

Isolated liver cells from starved clofibrate-treated rats were incubated for 40 min. Rotenone was present where indicated at 16 μM. Initial substrate concentrations were as follows: lactate, 10 mM; acetoacetate, 8 mM; [1-^{14}C]palmitate, 2 mM. Values for pyruvate represent the amount accumulated in the incubation vessel after 40 min, at which time [lactate]/[pyruvate] (*L/P*) was measured. Values for rates of metabolite uptake or formation are the mean ± SEM of 5–20 experiments

of pyruvate accumulation is a linear function of the log ([lactate]/[pyruvate]) and therefore possibly directly related to the free energy of the reaction [11]. Moreover, the various concentrations of DNP up to 75 μM generate a family of curves, lying parallel but to the right of the control curve. Effectively, this means that in the presence of increasing concentrations of DNP, a correspondingly higher ratio of [lactate]/[pyruvate] is found for any given rate of pyruvate accumulation by the cells. At the highest concentration of DNP used (100 μM) O_2-uptake and lactate removal were impaired (table 1) and this parallel relationship no longer held (fig.2). It is clear that the liver cell has a remarkable reserve capacity, allowing it to compensate for a major degree of energy wastage.

3.3. *Effects of rotenone and palmitate on the [lactate]/[pyruvate] ratio*

As an alternative method of impairing energy metabolism, liver cells were incubated with rotenone which inhibits NADH dehydrogenase [12]. The addition of rotenone strongly depressed oxidation of [1-^{14}C]palmitate to $^{14}CO_2$ and ketone bodies, and decreased respiration by >50%. The addition of 10 mM acetoacetate completely restored ketone body formation and brought about a partial recovery of O_2-uptake and $^{14}CO_2$ production. These effects were associated with a substantial conversion of the added acetoacetate to 3-hydroxybutyrate (table 2). The explanation of this phenomenon is straightforward. The NAD-linked hydroxyacyl CoA oxidation step of each loop of the fatty acid spiral is blocked by NADH accumulating as a consequence of the inhibition of NADH dehydrogenase by rotenone. Acetoacetate acts as a hydrogen sink for this NADH, allowing the oxidation of hydroxyacyl CoA to proceed. Because the flavin-linked dehydrogenation of acyl CoA circumvents the rotenone block, palmitate oxidation is largely restored. The flow of reducing equivalents from flavin to O_2 is coupled to phosphorylation so that levels of ATP, depressed by rotenone-treatment, are raised to ~60% of control values. Since oxidation of acetyl CoA derived from palmitate occurs to some extent, as evidenced by generation of $^{14}CO_2$, it appears that NAD-linked dehydrogenases of the tricarboxylic acid cycle can also couple with 3-hydroxybutyrate dehydrogenase to a limited extent, thereby circumventing the rotenone-induced block of cycle activity.

Table 2 also shows that the impairment of lactate uptake, induced by rotenone, was accompanied by a marked elevation of the [lactate]/[pyruvate] ratio. This rise was not prevented by added acetoacetate, the [lactate]/[pyruvate] ratio, in fact, increasing still further in its presence. Palmitate by itself, was also unable to prevent the elevation of the [lactate]/[pyruvate] ratio induced by rotenone. However, the combination of acetoacetate and palmitate promoted a gradual accumulation of substantial amounts of pyruvate, and reduced the [lactate]/[pyruvate] ratio to a value only twice that of cells incubated for 40 min with lactate and palmitate in the absence of inhibitor. The addition of acetoacetate caused a >8-fold increase in the formation of $^{14}CO_2$ from [1-^{14}C]palmitate in the presence of lactate and rotenone, whereas ketone body production remained essentially unchanged. Hence the lowering of the [lactate]/[pyruvate] ratio towards normal appears to have been associated with an enhancement of tricarboxylic acid cycle activity.

4. Discussion

In the experiments reported here, two different approaches have been adopted to demonstrate that the steady-state [lactate]/[pyruvate] ratio is sensitive to agents which interfere with energy metabolism. The action of DNP in raising the ratio is particularly remarkable in the face of the greatly enhanced O_2-uptake and increased removal of lactate induced by the uncoupler. Thus, the elevated ratio cannot be due to an inhibition of lactate oxidation to pyruvate. Nor do the changes in the [lactate]/[pyruvate] ratio merely reflect corresponding changes in mitochondrial redox state, since uncoupling agents are known to raise mitochondrial [NAD]/[NADH] [13]. The conclusion seems inescapable that energy is required to maintain the normal near-equilibrium steady-state value of the [lactate]/[pyruvate] ratio at ~8/1 and when energy metabolism is compromised the ratio rises. Thus, these responses of the [lactate]/[pyruvate] ratio to uncoupling agents imply that the hepatic cytoplasmic NAD(H) pool is held in a readily reversible steady-state by energy-dependent mechanisms.

The sensitivity of the [lactate]/[pyruvate] ratio to factors inhibiting energy metabolism was confirmed by treating cells with rotenone. The restoration of the ratio to values close to normal by the simultaneous addition of palmitate and acetoacetate is difficult to

explain other than on the basis that the fatty acid acted as a source of utilizable energy. The counteracting of the effects of palmitate by DNP (table 2) would seem to confirm this view. Even if some of the pyruvate formed was peroxisomal in origin (and this seems unlikely since clofibrate treatment depresses peroxisomal lactate oxidase [14]) palmitate would be expected to compete with lactate for peroxisomal oxidation rather than to enhance it [15]. Hence, it appears that the effects of palmitate were mediated by mitochondrial mechanisms in which the fatty acid acted as an energy source. This conclusion is also supported by the finding that substitution of antimycin for rotenone prevented both reduction of acetoacetate by palmitate, and pyruvate accumulation (not shown).

Krebs and co-workers have shown that near-equilibrium exists between the mitochondrial respiratory chain and the extramitochondrial phosphorylation state [16]. Further, a near-equilibrium relationship has been derived [17], linking the extramitochondrial phosphorylation state and redox state of the NAD couple as follows:

$$\frac{[ATP]}{[ADP][HPO_4^{2-}]} = \frac{[pyruvate]}{[lactate]} \cdot \frac{[glyceraldehyde\ 3\text{-}phosphate]}{[3\text{-}phosphoglycerate]} \cdot K$$

It is evident that in this expression, [pyruvate]/[lactate] and [glyceralde 3-phosphate]/[3-phosphoglycerate] can vary reciprocally, without altering the overall value of the right-hand side of the equation. Because of this a constant correlation between the extramitochondrial phosphorylation state and redox state of the NAD couple cannot be demonstrated [18], the value ($[NAD^+]/[NADH]$)/($[ATP]/[ADP][P_i]$) varying some 40-fold under different experimental conditions [18].

It has been suggested that when discrepancies such as this are observed, they can be explained on the basis of the existence of ion gradients or membrane potentials which perturb the chemical equilibria [16]. We interpret our data in accord with this proposal, and suggest that the shift in the apparent equilibrium of the lactate dehydrogenase reaction, brought about by energy input, is an example of a large class of energy-dependent redox reactions, associated with the process of 'reversed electron transfer' [13,19,20]. With the widespread acceptance of the principles embodied in the Mitchell chemiosmotic hypothesis [21–25], the view is gaining ground that reversed electron transfer is another facet of the same phenomenon [26]; that is, an electric field, brought about by charge separation, is able to drive a chemical reaction in an otherwise thermodynamically unfavourable direction [21,22]. An appropriate orientation of the redox carriers within the inner mitochondrial membrane would be necessary for this mechanism to function, and this has been shown to be the case for the energy-linked transhydrogenase [27,28].

There is considerable evidence that some hepatic lactate dehydrogenase may be intra-mitochondrial, or at least closely associated with these organelles [29,30], and that the shuttle enzyme malate dehydrogenase is a component of the inner membrane [31]. Lactate hydrogen appears to enter the mitochondria through the malate shuttle [4,5] completely separate from the shuttle transporting hydrogen derived from sorbitol [32]. Moreover, protons from lactate can be transferred to malate without exchanging with protons in cell water [33,34]. Thus, the whole apparatus for conveying reducing equivalents from lactate into the mitochondria may be closely associated with the mitochondrial membrane. Under these circumstances it is feasible that the proton generated during lactate oxidation to pyruvate may not be released immediately into the aqueous phase of the cell in a scalar manner, but may travel vectorially through hydrogen bonded relays within proteins [35,36] down a favourable gradient, under the influence of the electric field created by the charge separation reflected in the mitochondrial membrane potential. This process could effectively shift the equilibrium of the lactate dehydrogenase reaction in favour of pyruvate formation. DNP would bring about a rise in the steady-state [lactate]/[pyruvate] ratio by virtue of its action in diminishing the mitochondrial membrane potential [24]. It follows that estimates of the absolute value of the cytoplasmic [NAD]/[NADH] ratio (redox state) based on measurement of the [lactate]/[pyruvate] ratio may not be valid unless the possible contribution of an energy input reflected by the mitochondrial membrane potential is taken into account. In other words, for cellular redox reactions in which a proton is generated or consumed, the activity of the proton is a function not only of a concentration component but also of an electrical component.

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