FUTILE HYDROGEN CYCLING IN LIVER CELLS FROM TRIIODOTHYRONINE TREATED RATS

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Summary: Aminooxyacetate does not inhibit gluconeogenesis from sorbitol or glycerol, and ethanol does not inhibit gluconeogenesis from L-lactate, in liver cells prepared from triiodothyronine (T3) treated rats. These results are in accord with the previously documented marked increase in α-glycerol phosphate shuttle activity induced by thyroid hormones. Aminooxyacetate inhibits gluconeogenesis from L-lactate in hepatocytes from T3 treated rats by only about 30% (vs 90% in hepatocytes from normal rats). Also pyruvate kinase flux during gluconeogenesis from L-lactate is markedly increased in liver cells from fasted, T3 treated, rats.

The very large increase in mitochondrial α-glycerophosphate oxidase activity induced by treatment of animals with thyroid hormones (1-4) results in an increased capacity of the α-glycerophosphate shuttle to translocate reducing equivalents from the cytosol to the mitochondria (5-7). We have previously postulated (8-10) a cycle which can effect transfer of reducing equivalents from the mitochondria to the cytosol: pyruvate $\xrightarrow{cyt}$ pyruvate $\xrightarrow{mit\ ATP}$ oxalacetate $\xrightarrow{mit\ NADH}$ malate $\xrightarrow{mit}$ malate $\xrightarrow{cyt\ NADH}$ oxalacetate $\xrightarrow{cyt\ GTP}$ phosphoenolpyruvate $\xrightarrow{cyt\ ATP}$ pyruvate ^{cyt}, where mit denotes mitochondrial and cyt denotes cytosolic. (This will be referred to as the export cycle in this paper.) We have presented evidence that such outward hydrogen transfer can occur via this cyclic mechanism under unusual conditions, as when high concentrations of pyruvate are presented as sole substrate to isolated liver cells from fasted rats (10-12). Whether such cycling could ever occur with more physiological substrate mixtures remained to be investigated. In this

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paper we present evidence that, in liver cells from triiodothyronine (T3) treated rats, concurrent operation of shuttles transferring hydrogen from mitochondria to cytosol, and from cytosol to mitochondria, exists during gluconeogenesis from L-lactate.

METHODS: Isolated hepatocytes were prepared (from rats fasted for 24 hours) according to the procedure of Berry and Friend (13), modified by the use of Ca^{+2} free Krebs-Henseleit (14) buffer and the omission of hyaluronidase. Hyperthyroid animals were obtained by injecting 200 gm rats for three successive days with 100 μgm of triiodothyronine in 0.5 ml saline, the rats being used (after 24 hour fast) on either the fourth or fifth day after the first injection. The liver cells (.15 ml to .25 ml packed cells) were incubated in 2 to 4 ml of Krebs-Henseleit buffer (containing 2.5 mM $CaCl_2$) in 25 ml glass Erlenmeyer flasks at 38°C in a rotary shaker bath (150 rev/min) with 95% O_2/5% CO_2 in the gas phase. Glucose was determined enzymically with hexokinase and glucose 6-P dehydrogenase from L. mesenteroides, using NAD^+ as cofactor.

Pyruvate kinase flux was estimated by the simplified procedure described previously (11). $NaH^{14}CO_3$ was used as the labeled substrate, since its specific activity decreases only very slightly with time. The molar specific activity of phosphoenolpyruvate was calculated from one half that of the glucose formed, since essentially all of the glucose formed in liver cells from fasted rats comes from gluconeogenesis. Pyruvate kinase flux is somewhat underestimated, since no corrections were made as previously (11) for reutilization of pyruvate formed from phosphoenolpyruvate in the cell in the pyruvate carboxylase or pyruvate dehydrogenase reactions. As discussed previously (9, 12), the isotopic results also can overestimate pyruvate kinase flux to a small degree, mainly because malic enzyme (via a net malate ⟶ pyruvate reaction or via an isotopic exchange reaction) can also contribute to the labeling of pyruvate and lactate. We have shown elsewhere (12) that a malic enzyme inhibitor, 2,4 dihydroxybutyrate (15), only slightly depresses labeling of pyruvate and lactate from $NaH^{14}CO_3$ in liver cells from fasted rats.

RESULTS AND DISCUSSION

It has previously been shown that, in liver cells from normal rats, aminooxyacetate can partially inhibit the utilization of substrates which produce excess cytosolic NADH, e.g. from such substrates as ethanol, glycerol and various polyols (10, 16, 17). Aminooxyacetate is a transaminase inhibitor (18, 19), and these results suggested that a malate-aspartate shuttle plays a role in the transfer of reducing equivalents from the cytosol to the mitochondria in liver cells from normal animals. Table I shows that in liver cells from triiodothyronine treated

Table I. Effects of Aminooxyacetate and of Ethanol on Gluconeogenesis in Hepatocytes from Triiodothyronine (T3) Treated and Untreated Rats.

Liver cells from fasted rats (untreated or treated with 50 μgm Triiodothyronine / 100 gm body wt for 3 to 4 days) were incubated for 60 min. with the substrate shown at 10 mM. Glucose formation is given as the mean ± S.E. for the number of experiments given in parentheses.

Substrate	(No. of Expts)	Hormonal Treatment	Additions AOA (mM)	Additions Ethanol (mM)	Average Glucose Formation μmoles (gm wet wt.hr)	Average % Change due to Additions
Glycerol	(8)	None	0	0	59 ± 5	
Glycerol	(8)	None	0.2	0	28 ± 3	-53
Glycerol	(3)	T3	0	0	70 ± 8	
Glycerol	(3)	T3	0.2	0	68 ± 6	- 3
Sorbitol	(8)	None	0	0	118 ± 4	
Sorbitol	(8)	None	0.2	0	68 ± 4	-42
Sorbitol	(3)	T3	0	0	125 ± 9	
Sorbitol	(3)	T3	0.2	0	124 ± 7	- 1
Lactate	(10)	None	0	0	54 ± 4	
Lactate	(10)	None	0.2	0	5 ± 2	-91
Lactate	(6)	None	0	10	35 ± 5	-35
Lactate	(4)	T3	0	0	72 ± 7	
Lactate	(4)	T3	0.2	0	50 ± 3	-30
Lactate	(3)	T3	0	10	78 ± 6	+ 9

rats, aminooxyacetate does not inhibit sorbitol or glycerol utilization. Increasing the aminooxyacetate concentration to 2 mM also does not cause inhibition of uptake of these substrates, (unpublished experiments), ruling out a T3-induced increase in glutamate oxalacetate transaminase as the cause of the lack of effect of aminooxyacetate. These results are then in accord

with a greatly increased capacity of the α-glycerophosphate shuttle in livers of T3 treated rats, as previously demonstrated by Werner and Berry (7).

Another result indicative of an increased α-glycerophosphate shuttle is the lack of an inhibitory effect of ethanol on gluconeogenesis from L-lactate in liver cells from T3-treated rats (Table I). The inhibitory effect of ethanol on gluconeogenesis from L-lactate in normal animals has long been known (20), and has been related to the increased production of cytosolic NADH produced by the oxidation of ethanol in the alcohol dehydrogenase reaction (21).

We have previously shown that aminooxyacetate causes a profound (approximately 90%) inhibition of gluconeogenesis from L-lactate in kidney cortex segments (22) and in isolated liver cells (10) from fasted rats. These results and similar results from other laboratories (23-25) have provided strong confirmatory evidence for the proposal of Lardy et al. (26) that aspartate is the carrier by which the carbon skeleton of oxalacetate is transported from the mitochondria to the cytosol, when L-lactate is the gluconeogenic substrate. In Table I we show that, by contrast, aminooxyacetate causes only about a 30% inhibition of gluconeogenesis from L-lactate in liver cells from T3-treated rats. This result suggests that most of the carbon transfer from mitochondria to cytosol is now occurring in the form of malate outflow, with aspartate outflow now making up less than 1/3 of the carbon flux. Since the lactate dehydrogenase reaction already produces all the NADH required for the reductive glyceraldehyde-P dehydrogenase step of the pathway of gluconeogenesis from L-lactate, the reducing equivalents carried by malate from the mitochondria to the cytosol and which then generate NADH in

the cytosolic malate dehydrogenase reaction, are in excess of biosynthetic needs and must be returned to the mitochondria via the α-glycerophosphate shuttle. The net result of this is that a considerable amount of hydrogen transfer from the mitochondrial NADH level to the mitochondrial flavin level (of the respiratory chain) occurs by a system which generates no ATP. One might also note that this estimation of malate efflux does not indicate the total extent (see also below) of excess cytosolic NADH production. To the extent that pyruvate formed from L-lactate is oxidized to acetyl-CoA (and this pathway was not measured in the experiments of Table I), the NADH produced in the lactate dehydrogenase reaction is also in excess of the needs for gluconeogenesis.

In previous experiments, with pyruvate as substrate for liver cells from normal fasted rats, we have estimated pyruvate kinase flux by an isotopic technique, and have found some correlation between pyruvate kinase flux and the rate of lactate formation (11). When L-lactate is the gluconeogenic substrate for liver cells from normal fasted rats, the rate of pyruvate kinase flux is quite low, generally less than 10% of the rate of gluconeogenesis (27). However, as shown in Table II, when L-lactate is the substrate for liver cells from T3-treated (and fasted) rats, the rate of pyruvate kinase is nearly half that of the rate of glucose formation. Aminooxyacetate (0.2 mM) causes only a small decrease in pyruvate kinase flux, indicating that a cycle between pyruvate and phosphoenolpyruvate involving aspartate transfer (9, 28) is not the major pathway accounting for the pyruvate kinase flux. We are left with the conclusion that the pyruvate kinase flux in these liver cells from T3-treated rats reflects the operation of the export cycle which

Table II. Estimation of Pyruvate Kinase Flux During Gluconeogenesis from L-Lactate in Liver Cells from Triiodothyronine Treated Rats.

Liver cells from T3-treated and fasted rats were incubated with 20 mM L-lactate in 4 ml of Krebs-Henseleit buffer containing 30 to 50 μC of $NaH^{14}CO_3$.

Expt. No.	0.2 mM AOA	Isotopic Yields from $NaH^{14}CO_3$ (cpm X 10^{-6}) Glucose	Lactate + Pyruvate	Analytical Glucose Formation (μmoles/gm wet wt/hr)	Estimated Pyruvate Kinase Flux (μmoles/gm wet wt/hr)
1	-	1.512	.841	67	75
	+	1.018	.677	49	65
2	-	2.405	.910	80	61
3	-	2.116	1.109	75	79
4	-	2.392	1.163	72	70

effects a net transfer of reducing equivalents (with no net transfer of carbon) from the mitochondria to the cytosol. This is evidence that this cycle may operate during metabolism of an important physiological substrate, lactate, in liver cells from T3-treated animals. The results of Table II indicate that the malate outflow from the mitochondria is even larger than that calculated in the previous paragraph, since some of the carbon is recycled to pyruvate via pyruvate kinase and back into the mitochondria. To the extent that reducing equivalents are transferred from the mitochondria to the cytosol via the export cycle, and then returned to the mitochondria via the α-glycerophosphate shuttle, not only is there no ATP generation in the conversion of mitochondrial NADH to FH_2, but there is a net utilization of one ATP in the process, since the export cycle requires a net

input of one ATP per cycle. The simultaneous operation of cycles which transfer reducing equivalents in both directions between mitochondria and cytosol constitutes an apparent, energy-wasting, futile cycle. However, these cycles could contribute to the thermogenic action of the thyroid hormones.

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