PRELIMINARY REPORT

Triiodothyronine Treatment Increases Substrate Cycling Between Pyruvate Carboxylase and Malic Enzyme in Perfused Rat Liver

Kitt Falk Petersen, James B. Blair, and Gerald I. Shulman

The relative roles of pyruvate kinase and malic enzyme in substrate cycling between pyruvate and oxaloacetate were examined in perfused livers of 24-hour–fasted normal and triiodothyronine (T_3)-treated rats using an inhibitor of malic enzyme (hydroxymalonate). Livers were perfused for 60 minutes in a recirculating system with [3-^{13}C]alanine (10 mmol/L, 99% ^{13}C-enriched). The combined flux through pyruvate kinase plus malic enzyme relative to pyruvate carboxylase flux was assessed by the ^{13}C-enrichment ratio of alanine C2 to glucose C5 in the perfusate, determined with ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. In normal rat livers, the relative carbon flux through pyruvate kinase plus malic enzyme to pyruvate carboxylase was 0.18 ± 0.04, and increased to 0.44 ± 0.08 ($P < .05$) in the T_3-treated group. After addition of hydroxymalonate, this relative carbon flux was unchanged in normal rat livers, but decreased to 0.15 ± 0.04 ($P < .01$) in the T_3-treated group, suggesting that the increased carbon flux in T_3-treated livers was caused by increased flux through malic enzyme. Malic enzyme activity increased from 0.36 ± 0.05 U/g liver in normal livers to 2.51 ± 0.50 U/g liver ($P < .05$) in the T_3-treated group, whereas there was no effect of T_3 treatment on pyruvate kinase activity. We conclude that (1) carbon flux through malic enzyme relative to pyruvate carboxylase flux is minimal in the liver of normal 24-hour–fasted rats, (2) T_3 treatment stimulates substrate cycling between pyruvate and oxaloacetate by increasing carbon flux through malic enzyme, and (3) under hyperthyroid conditions, substrate cycling between pyruvate carboxylase and malic enzyme accounts for a major fraction of the gluconeogenic flux.

THREE SUBSTRATE CYCLES between the pathways of glycolysis and gluconeogenesis are known to occur in the liver: (1) the glucose ↔ glucose-6-phosphate cycle, (2) the fructose-6-phosphate ↔ fructose-1,6-diphosphate cycle, and (3) the phosphoenolpyruvate ↔ pyruvate cycle. Of these, the phosphoenolpyruvate ↔ pyruvate cycle is the most complex, since it involves three enzymes (pyruvate kinase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase [PEPCK]) located in different cellular compartments.[1] In addition, there is another potential substrate cycle between pyruvate and oxaloacetate catalyzed by pyruvate carboxylase and malic enzyme that to date has received little attention. Previous in vitro studies that have examined the phosphoenolpyruvate ↔ pyruvate cycle in both hepatocytes and perfused livers obtained from normal rats have found that pyruvate kinase flux ranged between 10% and 45% of the gluconeogenetic flux.[2-4] A threefold increase in the rate of this cycle was found to occur in hepatocytes and perfused livers obtained from triiodothyronine (T_3)-treated rats, which was attributed to increased activity of pyruvate kinase.[4,5] However, the methods used to assess substrate cycling between phosphoenolpyruvate and pyruvate in these studies could not distinguish between contributions made by pyruvate kinase (phosphoenolpyruvate ↔ pyruvate) versus malic enzyme (malate ↔ pyruvate). In the present study, we examined the respective roles of pyruvate kinase and malic enzyme in substrate cycling between pyruvate and oxaloacetate in normal and T_3-treated rats using a specific inhibitor of malic enzyme (hydroxymalonate). Substrate cycling between pyruvate and oxaloacetate was examined in a recirculating perfused-liver preparation using [3-^{13}C]alanine as both the substrate and the trapping pool, in which the combined flux through pyruvate kinase and malic enzyme relative to the carbon flux through pyruvate carboxylase was estimated by examining the ratio of ^{13}C incorporated into the C2 position of alanine relative to that in the C5 position of glucose in the perfusate, using both ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.[6,7]

MATERIALS AND METHODS

Reagents

L-[3-^{13}C]alanine was purchased from Tracer Technologies (Somerville, MA). 3,3′-5-Triiodo-L-thyronine sodium salt, bovine albumin, and tartronic acid (hydroxymalonic acid) were purchased from Sigma Chemical (St Louis, MO).

Animals

Ten male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) with an initial body weight of 330 ± 18 g (mean ± SE) were given T_3 (5 mg 3,3′-5-triiodo-L-thyronine sodium salt dissolved in 300 μL 0.1-mol/L NaOH per liter of water) in the drinking water for 8 days before the experiment. During that period, rats lost 36 ± 10 to 293 ± 9 g despite normal intake of food and water. The control group consisted of 12 normal rats weighing 325 ± 19 g.

Experimental Design

All rats were fasted for 24 hours to deplete liver glycogen,[8] and after anesthesia with pentobarbital (50 mg/kg intraperitoneally),

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the liver was isolated for perfusion as described previously.[9] An initial washout period of 15 minutes' perfusion with a non-recirculating perfusate (Krebs-Henseleit bicarbonate buffer with 1% bovine albumin, pH 7.45, 37°C, at a flow rate of 40 mL/min) preceded the 60-minute experimental period in which the liver was perfused with 50 mL recirculating perfusate consisting of Krebs-Henseleit buffer with 10 mmol/L [3-^{13}C]alanine (99% ^{13}C-enriched). Malic enzyme was inhibited with the addition of hydroxymalonate (10 mmol/L) to the perfusate. At the end of the experiment, approximately 1 g of the liver was placed on an aluminum spatula precooled to −30°C on dry ice for determination of enzymatic activities of pyruvate kinase and malic enzyme and triglyceride concentration. The remaining liver tissue was freeze-clamped in liquid nitrogen for determination of glycogen, and the perfusate was collected for ^{13}C and ^{1}H NMR spectroscopy. All samples were stored at −70°C until analyses.

Analyses

Perfusate glucose concentration was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Liver glycogen concentration was determined in 120 to 200 mg liver tissue.[10] Lipids were extracted from liver tissue with chloroform/methanol,[11] and triglyceride concentration was determined with a commercial kit (Sigma Chemical, St Louis, MO). Pyruvate kinase activity and malic enzyme activity were determined as previously described.[12-14]

^{13}C and ^{1}H NMR Spectroscopy

Relative ^{13}C enrichments in each carbon position of glucose and alanine were determined using ^{13}C NMR. The perfusate was dried, taken up in 0.6 mL deuterium oxide, and filtered, and ^{13}C NMR spectra were obtained at 25°C in a spectrometer (Bruker WM 500 Spectrometer; Bruker Instruments, Billerica, MA) operating at 125.76 MHz.[8,15] Glucose was isolated for ^{1}H NMR spectroscopy as previously described, and fractional ^{13}C enrichment of glucose C1 was determined by the relative ratio of the satellites to the total peak area in the ^{1}H NMR spectrum.[8] The ^{13}C enrichments of individual carbon atoms of glucose were calculated as the product of fractional ^{13}C enrichments and relative ^{13}C enrichments determined by ^{13}C NMR spectroscopy.[7] The ^{13}C enrichment of alanine C2 was calculated by multiplying the relative enrichments of C2 to C3, with ^{13}C enrichment of C3 assigned as 99%.

Calculation of Flux Rates of Malic Enzyme, Pyruvate Kinase, and Pyruvate Carboxylase, and Glucose Production

From [3-^{13}C]alanine, ^{13}C is transferred via [3-^{13}C]pyruvate into the C3 position of oxaloacetate via pyruvate carboxylase to form [3-^{13}C]oxaloacetate (Fig 1). Due to fumarase equilibration in the Krebs cycle, ^{13}C is scrambled into [2,3-^{13}C]oxaloacetate. The labeled C2 enters phosphoenolpyruvate via PEPCK to form [2,3-^{13}C]phosphoenolpyruvate, which leads to formation of glucose labeled as [1,2,5,6-^{13}C]glucose, in which C1 ≈ C6 as C1 and C6 are from C3 phosphoenolpyruvate and C2 ≈ C5 as C2 and C5 are from the scrambled C2 in phosphoenolpyruvate. Due to pyruvate kinase but also to malic enzyme, pyruvate is recycled from [2,3-^{13}C]phosphoenolpyruvate as [2,3-^{13}C]pyruvate. This is schematically shown in Fig 1. When [2,3-^{13}C]oxaloacetate enters the pathway of fatty acid synthesis, [2,3-^{13}C]malate is formed and converted to [2,3-^{13}C]pyruvate via malic enzyme; therefore, ^{13}C enrichment in pyruvate C2 reflects carbon flux not only through pyruvate kinase but also through malic enzyme (Fig 1). In the presence of a large trapping pool of [3-^{13}C]alanine, the ratio of ^{13}C in alanine C2 relative to ^{13}C in glucose C5 is a measure of the combined carbon flux through pyruvate kinase plus malic enzyme relative to pyruvate carboxylase flux. The rate of flux through pyruvate kinase plus malic enzyme can be calculated as the rate of glucose production multiplied by the ratio of ^{13}C enrichment in alanine C2 to glucose C5 times 2. When malic enzyme is fully inhibited, the ^{13}C label incorporated into alanine C2 will be only due to carbon flux through pyruvate kinase.

Statistical Analysis

Groups were compared using Student's *t* test or ANOVA where appropriate. Statistical significance was assumed when *P* was less than .05. All data are expressed as the mean ± SE.

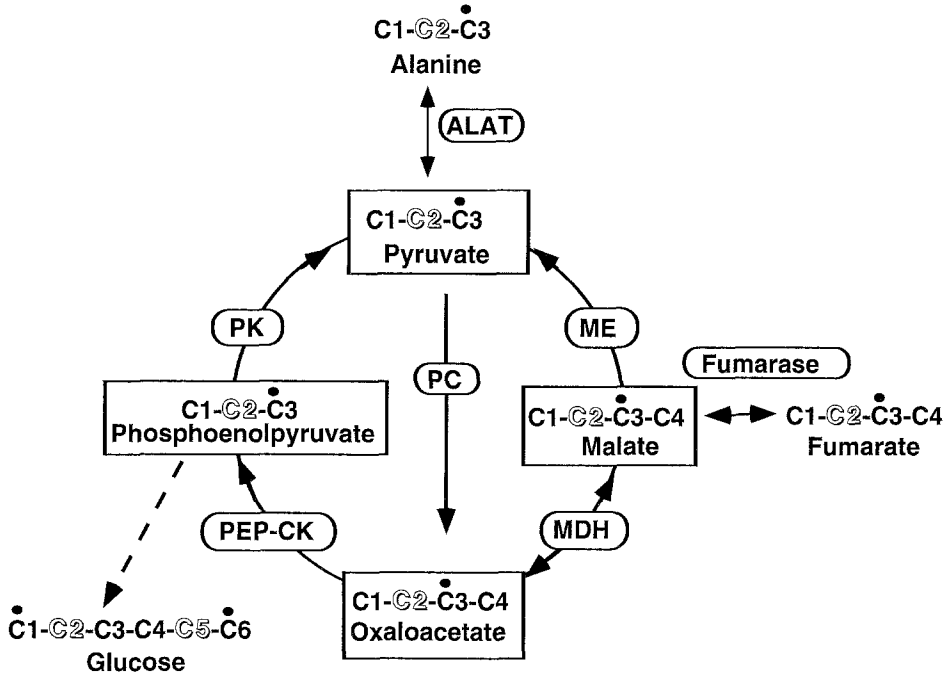

Fig 1. Major pathways of the substrate cycles involving recycling of pyruvate through pyruvate kinase and malic enzyme. C, carbons labeled from the primary ^{13}C; ℂ, carbons labeled with ^{13}C due to scrambling. ALAT, alanine aminotransferase; PK, pyruvate kinase; ME, malic enzyme; MDH, malate dehydrogenase; PC, pyruvate carboxylase.

RESULTS

The mean ^{13}C enrichment (atom percent excess above 1.1% natural abundance) of the individual carbons of glucose and alanine from the four groups is shown in Table 1. ^{13}C enrichments of C1 and C6 were similar in the four groups, but ^{13}C enrichment of C2 and C5 were significantly lower in the T_3-treated group than in the normal control group ($P < .05$). ^{13}C from oxaloacetate is scrambled into glucose C2 and C5, and to the extent that fumarate and malate are not fully equilibrated in the tricarboxylic acid cycle, ^{13}C enrichment for C2 and C5 will be less than for C1 and C6. Ratios of C1 to C2 and C6 to C5 were significantly higher in the T_3-treated group than in control (C1/C2, 1.87 *v* 1.34 [$P < .05$]; C6/C5, 1.93 *v* 1.41 [$P < .05$]), suggesting less equilibration between fumarate and malate due to relatively increased carbon flux through pyruvate carboxylase relative to malate/fumarate equilibration after T_3 treatment, respectively.

In experiments with livers from normal rats, the ^{13}C enrichment in alanine C2 relative to glucose C5 was 0.18 ± 0.04 and no significant change occurred with the addition of hydroxymalonate (ratio of alanine C2 to glucose C5, 0.13 ± 0.03), indicating that recycling of pyruvate in normal 24-hour–fasted livers is mainly due to flux through pyruvate kinase. In the T_3-treated group, the ratio of ^{13}C enrichment in alanine C2 to glucose C5 increased to 0.44 ± 0.08 ($P < .01$ *v* normal), reflecting increased pyruvate cycling. When hydroxymalonate was added, this ratio decreased to normal values (0.15 ± 0.04, $P < .05$ *v* T_3-treated rats without inhibitor; Table 1). The reduction in the ratio of ^{13}C enrichment in alanine C2 to glucose C5 suggests a major role for malic enzyme in substrate cycling between pyruvate and oxaloacetate in livers from T_3-treated rats. Liver glycogen stores were almost totally depleted in both groups following the 24-hour fast (~0.22 g/100 g liver), so the rates of glucose production can be attributed almost entirely to gluconeogenesis. The rate of glucose production was not affected by hydroxymalonate: 3.3 ± 0.3 and 2.4 ± 0.1 μmol/g liver · min in normal and T_3-treated livers before hydroxymalonate and 3.7 ± 0.2 and 1.9 ± 0.2 μmol/g liver · min after hydroxymalonate, respectively. The rate of cycling between pyruvate and oxaloacetate in normal rat livers accounted for 26% to 36% of the rate of glucose production and can be attributed almost entirely to pyruvate kinase. In livers from T_3-treated rats, the rate of carbon cycling between pyruvate and oxaloacetate increased approximately to 88% of the rate of glucose production. After inhibition of malic enzyme, the rate of pyruvate to oxaloacetate cycling decreased to 30%; thus, approximately 50% of the carbon cycling between pyruvate and oxaloacetate in the hyperthyroid state can be attributed to carbon cycling through malic enzyme.

Activity in the pentose shunt will cause dilution of C2 in [1,2,5,6-^{13}C]glucose as [1,4,5-^{13}C]pentose-5-phosphate is formed and in the sequential actions of transketolase, transaldolase, and transketolase two molecules of fructose-6-phosphate labeled in the 1, 3, 5, and 6 positions are formed; thus, activity of the pentose phosphate cycle will be reflected by the difference between C1/C2 and C6/C5 ratios of ^{13}C enrichment in [1,2,5,6-^{13}C]glucose. In both normal and T_3-treated livers, ^{13}C enrichment of C1 relative to C2 was approximately 5% less than the relative ^{13}C enrichment of C6 to C5, suggesting that the relative flux of glucose through the pentose phosphate pathway was less than 5% of the rate of gluconeogenesis under both euthyroid and hyperthyroid conditions (Table 1).

T_3 treatment did not change the total enzyme activity of pyruvate kinase in liver tissue (control, 15.38 ± 5.72 U/g liver; T_3, 10.08 ± 1.28 U/g liver) but significantly increased malic enzyme activity, as previously described[7] (normal, 0.36 ± 0.05; T_3-treated, 2.64 ± 0.62; $P < .01$). Malic enzyme is a lipogenic enzyme that provides NADPH for triglyceride synthesis; however, there was no significant difference between triglyceride concentrations in liver tissue from T_3-treated and control animals (T_3-treated, 1.11 ± 0.76 mg/g wet weight liver [n = 6]; normal, 1.97 ± 1.41 mg/g wet weight liver [n = 10]).

DISCUSSION

There is currently little information about the role of malic enzyme in substrate cycling between malate and pyruvate. Rognstad[16] used 2,4-dihydroxybutyrate to inhibit malic enzyme in hepatocytes from normal rats incubated with 20 mmol/L L-lactate and $NaH^{14}CO_3$ to study the relative rates of carbon flux through pyruvate kinase plus malic enzyme and through PEPCK. In this approach,

Table 1. Percent ^{13}C Enrichment in Glucose C1 Through C6 and Alanine C2 and the Ratio of Alanine C2 to Glucose C5 in Liver Perfusate

Group	Glucose C1	Glucose C2	Glucose C3	Glucose C4	Glucose C5	Glucose C6	Alanine C2	Ratio of Alanine C2 to Glucose C5
Control (n = 6)	21.2 ± 2.6	15.8 ± 2.2	4.1 ± 0.4	3.8 ± 0.6	14.3 ± 1.9§	20.2 ± 3.1	2.6 ± 0.8	0.18 ± 0.04
Control + hydroxymalonate (n = 6)	20.5 ± 3.0	16.4 ± 2.4	3.3 ± 0.6	3.3 ± 0.7	16.5 ± 2.9‖	18.4 ± 2.6	1.9 ± 0.2	0.13 ± 0.03
T_3-treated (n = 6)	19.6 ± 5.0	10.5 ± 1.6†	4.0 ± 1.3	3.8 ± 0.8	10.4 ± 1.0¶	20.1 ± 4.9	4.4 ± 0.5*	0.44 ± 0.08*
T_3-treated + hydroxymalonate (n = 4)	20.3 ± 3.6	13.5 ± 2.0†	4.8 ± 0.6	4.8 ± 0.5	12.9 ± 1.7¶	20.8 ± 3.6	1.7 ± 0.3**	0.15 ± 0.04††

*$P < .01$ *v* control.
†$P < .05$ *v* C1.
§$P < .01$, ‖NS, ¶$P < .005$: *v* C6.
**$P < .01$ *v* T_3-treated.
††$P < .05$ *v* T_3-treated.

specific activity of phosphoenolpyruvate was estimated as half the ^{14}C specific activity of glucose, and pyruvate kinase flux as the amount of ^{14}C trapped in the pyruvate (and lactate) pool divided by the specific activity of phosphenolpyruvate. The relative rate of flux through pyruvate kinase to pyruvate carboxylase was estimated to be 10%, and no effect of the malic enzyme inhibitor, 2,4-dihydroxybutyrate, was found. This is consistent with our observations and suggests that malic enzyme plays a minor role in pyruvate cycling in the normal fasted rat liver. In a later study, Rognstad[5] found carbon cycling in hepatocytes from T_3-treated rats to be approximately 50% of the rate of gluconeogenesis, and although no inhibitor of malic enzyme was used to distinguish between cycling through pyruvate kinase and malic enzyme, he concluded that this increase was caused mainly by increased pyruvate kinase flux. Most recently, Cohen et al[4] examined the relative roles of pyruvate kinase and malic enzyme in causing the increased cycling of substrate between pyruvate and oxaloacetate observed in T_3-treated animals, using 2,4-dihydroxybutyrate in a single experiment in hepatocytes obtained from T_3-treated rats. In this study, incorporation of ^{13}C into alanine C2 and glucose C5 was assessed using ^{13}C NMR spectroscopy, and no effect of malic enzyme inhibition on pyruvate substrate cycling was found. It is unclear why the results of Cohen et al[4] are different from ours, although 2,4-dihydroxybutyrate is a much weaker competitive inhibitor than hydroxymalonate, which was used in our experiments.[17]

In summary, combined flux through pyruvate kinase plus malic enzyme was found to be 26% to 37% of the flux through pyruvate carboxylase in normal livers. Addition of hydroxymalonate had no effect on these flux rates, suggesting that carbon cycling in this state can be mostly attributed to pyruvate kinase activity. With T_3 treatment, this carbon flux increased to 88% of pyruvate carboxylase flux and normalized after inhibition of malic enzyme, suggesting that the increased substrate cycling between pyruvate and oxaloacetate was due to increased carbon cycling through malic enzyme. In accordance with these findings, the enzyme activities of pyruvate kinase were unchanged by T_3 treatment, whereas the activity of malic enzyme was increased sevenfold. In conclusion, under hyperthyroid conditions, substrate cycling between pyruvate carboxylase and malic enzyme is substantial and accounts for a major fraction of the gluconeogenic flux. This increased carbon cycling between pyruvate carboxylase and malic enzyme may contribute to the hypermetabolic state of hyperthyroidism.

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