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Befroy *et al.* reply

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Burgess *et al.*[1] comment that the modeling approach used in our recent article[2] may have led to an incorrect estimate of hepatic anaplerosis because we did not explicitly include a term for pyruvate cycling. Although we agree that a pyruvate substrate cycle is a feature of hepatic metabolism, we contend that under most conditions, the flux through this cycle is low, relative to the TCA cycle (V_{TCA}), and therefore does not substantially affect the rates of anaplerosis (V_{ANA}) that we reported. Furthermore, our measurements of V_{TCA} depend primarily on the kinetics of enrichment of glutamate at C5, which are independent of V_{ANA} and pyruvate cycling via pyruvate kinase (V_{PK}). Several independent lines of evidence support these conclusions.

Hepatic glutamate enrichment during these experiments is a complex function of the enrichments in acetyl-CoA, pyruvate and carbon dioxide, and the metabolic reactions that interact with these substrates, including V_{TCA}, gluconeogenesis from phosphoenolpyruvate and entry of pyruvate–carbon-dioxide through pyruvate carboxylase and malic enzyme. Under fasting conditions, malic enzyme activity is low[3,4], therefore pyruvate entry is almost exclusively through pyruvate carboxylase flux (V_{PC}). V_{PC} incorporates components owing to V_{ANA} and V_{PK}, which we consider to be metabolically distinct processes. Anaplerotic pyruvate entry is required under gluconeogenic conditions to replenish the pool of TCA cycle intermediates that would otherwise be depleted due to the consumption of oxaloacetate (OAA) to form glucose via phosphoenolpyruvate (PEP), whereas pyruvate cycling involves

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the futile consumption and regeneration of pyruvate. Under this rationale, $V_{PC} = [V_{ANA} + V_{PK}]$. Because the total metabolite mass remains constant, V_{ANA} is also equivalent to the gluconeogenic flux from PEP. An earlier version of our model of hepatic metabolism included a discrete term for V_{PK} to account for pyruvate cycling. Probability distribution analysis of Monte Carlo simulations of this model determined that V_{PK}/V_{TCA} was most likely to be <<0.49, with upper bound estimates (the 66th and 95th percentile of all runs) of 0.99 and 2.19, well below the values reported by Burgess *et al.*[5,6]. More importantly, simulations demonstrated that pyruvate cycling had no significant impact on our estimates of V_{ANA} and V_{TCA}. Although our analysis indicated that PK flux must be low, owing to uncertainty in establishing an absolute rate of pyruvate cycling we felt that including it in our article would be misleading.

In support of their hypothesis, Burgess *et al.*[1] applied a steady-state model[7] to simulate the ratio of glutamate C1 to glutamate C5 under a range of PK flux conditions. Unfortunately, their model is not suitable for examining our data because the system was not at isotopic steady state—enrichment of bicarbonate and glutamate C1 continue to increase throughout the 2-h study—and this leads to erroneous interpretations. In contrast, our analysis is based on the kinetics of plasma acetate and hepatic bicarbonate and glutamate ^{13}C enrichment, where each time point contributes to establishing the metabolic rates that best fit the raw data. Here we demonstrate the effects of pyruvate cycling on the kinetics and magnitude of glutamate C1 enrichment (Fig. 1); low rates of V_{PK} clearly fit the experimental data better than the higher values ($V_{PK}/V_{TCA} > 3.5$) suggested by Burgess *et al.*[1,5,6].

Low rates of pyruvate cycling are supported by other experimental evidence. We observed minimal labeling at C1 of pyruvate in liver tissue extracts from our studies of rats infused with [1-^{13}C] acetate[2], suggesting that PK flux is very low. We have also estimated $V_{(PK + ME)} / V_{(PC + PDH)}$ from the ratio of alanine C2 to glucose C5 enrichment in rat liver during an infusion of [3-^{13}C]alanine[3,4]. Hepatic $V_{(PK + ME)}$ in these experiments was estimated to be <27% of $V_{(PC + PDH)}$, more than an order of magnitude lower than that reported by Sunny *et al.*[5]. Furthermore, during an infusion of [3-^{13}C] lactate in humans who had fasted overnight, we observed negligible ^{13}C labeling of C2 in hepatic alanine and C2 of lactate pools using our *in vivo* ^{13}C magnetic resonance spectroscopy technique (Fig. 2). The shift in ^{13}C label from the C3 to the C2 position requires an isotopic equilibration that occurs owing to the rapid interconversion of OAA-malate-fumarate followed by the regeneration of pyruvate via V_{PK}. Rapid rates of pyruvate cycling would lead to the appearance of alanine C2 and lactate C2 peaks of similar magnitude to their C3 counterparts, which we did not observe (Fig. 2). In contrast, we observed substantial ^{13}C enrichment of hepatic glutamate C2 and glucose C6 owing to anaplerosis and gluconeogenesis, respectively.

Finally, pyruvate cycling and gluconeogenesis from PEP require obligate energy consumption. On the basis of the data from Sunny *et al.*[5], where gluconeogenesis is approximately equivalent to TCA flux and pyruvate cycling is ~3.5 times TCA flux, the energy produced by the TCA cycle would be almost entirely consumed by these processes, a situation that is energetically unsustainable.

In conclusion, the rates of pyruvate cycling reported by Burgess *et al.*[1,5,6] are incompatible with our human data[2] and inconsistent with animal studies that have directly measured this flux *in vitro*[3,8,9] and *in vivo*[4]. This difference may arise because [U-^{13}C]propionate, the tracer used by Burgess *et al.*[5,6], probably promotes pyruvate cycling anaplerosis, and gluconeogenesis by mass action effects and/or by its rapid conversion to propionyl-CoA and subsequent activation of pyruvate carboxylase[10]. Future studies should be able to assess this possibility directly.

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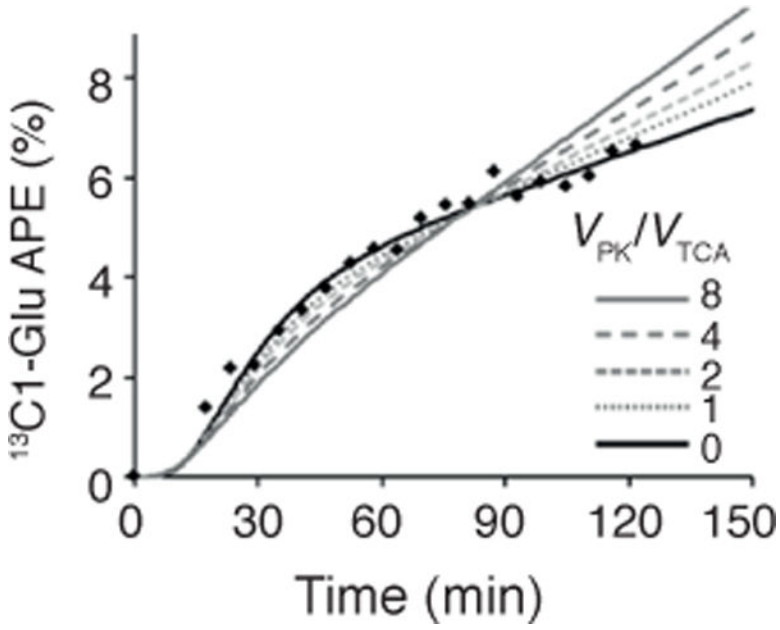

Figure 1.

The effect of pyruvate cycling, expressed as V_{PK}/V_{TCA}, on the kinetics of hepatic [1-^{13}C]glutamate (^{13}C1-Glu) enrichment during a 2-h infusion of [1-^{13}C]acetate. Raw data (black diamonds) are reproduced from Befroy *et al.*[2]. Low rates of V_{PK} clearly fit the experimental data better than the higher values suggested by Burgess *et al.*[1]. All studies were approved by the Yale Human Investigation Committee, and informed written consent was obtained from all subjects.

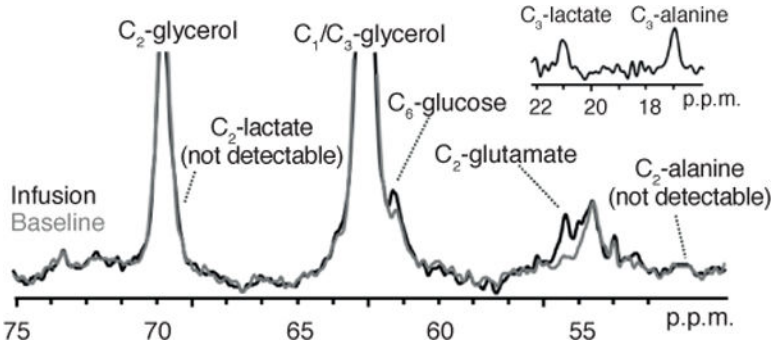

Figure 2.

^{13}C spectra acquired from human liver at baseline (gray) and after an infusion of [3-^{13}C]lactate (black). Negligible enrichment at alanine C2 and lactate C2 compared to their C3 counterparts (inset, acquired during the same study under identical conditions and shown at the same scale) indicate that V_{PK}/V_{TCA} is low. All studies were approved by the Yale Human Investigation Committee, and informed written consent was obtained from all subjects.