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report

Glutamine cycling in isolated working rat heart

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Cohen, David M., Patrick H. Guthrie, Xiaolian Gao, Ryosei Sakai, and Heinrich Taegtmeyer. Glutamine cycling in isolated working rat heart. *Am J Physiol Endocrinol Metab* 285: E1312–E1316, 2003. First published August 26, 2003; 10.1152/ajpendo.00539.2002.—To what extent does glutamine turnover keep pace with oxidative metabolism in the rat heart? To address this question, the following groups of substrates were presented to the isolated, working rat heart: *1*) glucose (5 mM), insulin (40 μU/ml), and [2-^{13}C]acetate (5 mM; high workload, $n = 5$); *2*) pyruvate (2.5 mM) and [2-^{13}C]acetate (5 mM; normal workload, $n = 5$); or *3*) propionate (1 mM) and [2-^{13}C]acetate (2.5 mM; normal workload, $n = 3$). In a subset of these experiments, the exchange of glutamate and glutamine was quantified by separation with ion exchange chromatography and analysis by GC-MS. There was an apparent equilibration of mass isotopomers of glutamate and glutamine after 50 min of perfusion, although the extent of equilibration was not determined. The fractional enrichment in glutamine was 31% of the enrichment of glutamate with the three different perfusates. From high-resolution nuclear magnetic resonance spectra, we found a ratio of glutamine to glutamate content of 94.1, 53.4, and 96.9%, respectively, for each experimental group. In experiments for which L-[1-^{13}C]glutamine (5 mM) was included in the perfusate of *group 2*, [1-^{13}C]glutamine was detected in the heart, but transfer of ^{13}C from glutamine to glutamate was not detected ($n = 4$). We conclude that, in the perfused working heart, production of glutamine by amidation of glutamate takes place and can be detected, whereas the reverse process, generation of glutamate from glutamine, remains undetected.

perfused heart; turnover; nuclear magnetic resonance; anaplerosis

THE METABOLIC ROLE OF GLUTAMINE in amino acid turnover and nitrogen balance is well appreciated (10), but the significance of glutamine in the heart is not well understood. Sources of cardiac glutamine are several: uptake from the circulation (9), proteolysis, and synthesis by glutamine synthetase. Glutamine synthetase is present in rat heart (2, 5, 13, 19), and so is glutaminase, the enzyme that degrades glutamine (8, 14).

The experimental model of heart metabolism has a substantial impact on the detection of glutamine turnover. In vivo studies have demonstrated incorporation of [^{13}N]ammonia into glutamine (11) (with positron emission tomography), as well as transfer of ^{13}C from glutamate to glutamine (20) [with an implanted radiofrequency probe and nuclear magnetic resonance (NMR) spectroscopy]. In the Langendorff model of the isolated perfused rat heart, a 10–30% decline in glutamine mass and negligible activity of conversion of glutamate to glutamine have been reported (3). Kinetic models of ^{13}C flow within the citric acid cycle typically include the glutamate pool but omit the glutamine pool (12), presumably owing to the latter's slow rate of turnover. Alternatively, the working model of the isolated perfused rat heart couples metabolic activity with performance of hydraulic work (18). This model exhibits greater metabolic rates than the Langendorff model and may expose higher rates of efflux from the citric acid cycle.

In the present communication, we report results in the working rat heart model that suggest the participation of glutamine in an energy-consuming substrate cycle. We observed the amidation of glutamate under several different experimental conditions but did not detect the deamidation of glutamine.

MATERIALS AND METHODS

Heart perfusion. Standard chemical reagents were obtained from Sigma. Sodium [2-^{13}C]acetate (99% enriched) was obtained from Cambridge Isotope Laboratories (Andover, MA). Male Sprague-Dawley rats (350–450 g) were provided free access to food and water and then anesthetized with pentobarbital sodium (15 mg/100 g body wt ip). The heart was rapidly removed and perfused in the working heart mode as described previously (18). Hearts were challenged with a preload of 15 cmH_2O and an afterload of either 100 cmH_2O ("normal" workload) or 140 cmH_2O ("high" workload). Heart rate, aortic pressure, and cardiac output were monitored continuously. Cardiac work was measured by multiplying the mean aortic pressure by the cardiac output. The 200 ml of recirculated perfusate contained substrates (listed below) and/or hormones (insulin), as well as Ca^{2+} (2.5 mM) and defatted bovine serum albumin (Pentex fraction V, 1% wt/vol) in Krebs-Henseleit buffer that was gassed with 95% O_2-5% CO_2. After 50 min of perfusion in the working mode, hearts were freeze-clamped with aluminum tongs cooled in liquid nitrogen and stored at −73°C.

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Table 1. *Experimental design*

Group	*n*	Sodium [2-^{13}C]Acetate	Substrates	Workload
1	5	5 mM	Glucose, 5 mM Insulin, 40 μU/ml	High
2	5	5 mM	Pyruvate, 2.5 mM	Normal
3	3	2.5 mM	Propionate, 1 mM	Normal
4	4	0	Sodium acetate, 5 mM Pyruvate, 2.5 mM L-[1-^{13}C]glutamine, 5 mM	Normal

Three groups of hearts were perfused with substrates and workloads shown in Table 1. In a subset of these experiments, the exchange of glutamate and glutamine was quantified by separation with ion exchange chromatography and analysis by gas chromatography-mass spectrometry.

Sample preparation. The frozen heart was ground to a fine powder, to which was added 3.6% perchloric acid (2 ml/g tissue). After centrifugation for 15 min (3,000 rpm at 5°C), the supernatant was retained and adjusted to pH 7 with 30% KOH. The sample was centrifuged again for 20 min (3,000 rpm at 5°C). The supernatant was retained and evaporated (Savant A290 speedvac). For subsequent analysis with NMR spectroscopy, the exchangeable protons were removed by adding 1.0 ml of $^2H_20$ (99% enriched; Cambridge Isotopes), drying, and repeating this procedure.

Gas chromatography-mass spectrometry. For analysis with gas chromatography-mass spectrometry after the NMR measurements were completed, samples were twice rinsed with 1.0 ml of H_2O and evaporated to remove the exchangeable deuterium atoms. Then 0.5 ml of 1 M acetic acid was added to a 0.7-ml sample and applied to a 1-ml bed volume cation exchange column (Dowex 50Wx8, H^+ form; Bio-Rad, Hercules, CA). The column was washed with 50 ml of water and eluted with 2 ml of 3 M ammonium hydroxide and 1 ml of water. After the eluent was collected and the sample evaporated, 500 μl of water and 5 μl of ammonium hydroxide were added. The sample was then applied to a 1-ml column of Dowex 1x8 (Cl^- form). Addition of 3.5 ml of water yielded the glutamine fraction, which was quickly evaporated. After a washing with 50 ml of water, the column was eluted with 3.0 ml of 1 M acetic acid and 1.0 ml of water, yielding the glutamate fraction, which was evaporated in the Savant.

Both the glutamate and the glutamine fractions were esterified with 1-propanol, derivatized with heptafluorobutyric acid, and evaporated gently under nitrogen gas. Glutamine yields the same derivative as glutamate due to heat-induced deamidation during esterification (6, 17). Each sample was taken up in 400 μl of ethyl acetate and stored at −20°C until separated with gas chromatography (Hewlett-Packard 5989B, HP5 column) and analyzed with mass spectrometry (Hewlett-Packard 5890 Series II). Negative ions were generated by methane chemical ionization, and the selected ions (mass-to-charge ration = 407–413) were monitored for the measurement of ^{13}C incorporation into glutamate and glutamine. The isotopic enrichment of each isotopomer was calculated as the difference in the isotopic distribution between the sample and natural standard (1).

High-resolution NMR spectroscopy. NMR spectroscopy was performed at 25°C in heart extracts dissolved in 0.7 ml of ^2H_2O (99% enriched, Cambridge Isotopes). Direct detection of ^{13}C (150.921 MHz) with proton decoupling was accomplished on a Bruker AM 600 NMR spectrometer (45° flip angle, interpulse delay of 1.0 s, sweep width of 254 ppm, 64K digitized data points per spectrum, and 6K free induction decays per spectrum). Spectra were processed with a line broadening of 0.0–1.0 Hz to enhance the signal-to-noise ratio. Several spectra were acquired without nuclear Overhauser enhancement (nOe) and with full relaxation (interpulse delay of 22.0 s) to correct for nOe and partial saturation in estimation of the areas of resonance peaks.

Calculations. High-resolution NMR spectra were used to estimate the molar ratio of [2-^{13}C]glutamine to [2-^{13}C]glutamate (more precisely, the molar ratio of positional isotopomers of glutamine possessing ^{13}C at carbon C2 to the corresponding isotopomers of glutamate). Estimates of molar ratios of [3-^{13}C]glutamine to [3-^{13}C]glutamate and of [4-^{13}C]glutamine to [4-^{13}C]glutamate were undertaken as well. The ratio of mass of glutamine to mass of glutamate was estimated from these molar ratios divided by the ratio of fractional enrichment of glutamine to glutamate. Relative anaplerosis "*y*" (the ratio of anaplerotic flux to the flux through citrate synthase) was estimated from the C3/C4 of glutamate by use of the formula $C3/C4 = 1/(1 + 2 \cdot y)$ (Ref. 4).

RESULTS

Cardiac function. Cardiac function was stable, and cardiac power was maintained during the 50 min of perfusion either at 13–14 mW (*group 1*, high workload) or at 7–11 mW (*groups 2* and *3*, normal workload) (Fig. 1). The data are consistent with earlier data on cardiac work from the laboratory (18).

Glutamine-to-glutamate ratio. Glutamine-to-glutamate molar ratios were 94–97% in the hearts perfused either with [2-^{13}C]acetate and glucose and insulin or with propionate (Table 1). However, in hearts perfused with [2-^{13}C]acetate and pyruvate, the glutamine-to-glutamate molar ratio fell to 53%, significantly different from the two other groups ($P < 0.05$).

Mass isotopomer analysis. The mass isotopomers of glutamate and glutamine were highly correlated (ρ = 96.8%), consistent with isotopic steady state (Fig. 2). The ratio of fractional enrichments of glutamine to glutamate was 31%, suggesting that 69% of the glutamine pool came from sources other than glutamate or that several pools of glutamine exist within the heart, only some of which are metabolically active.

However, it is possible that the conversion of glutamate to glutamine was not accompanied by deamidation of glutamine in our preparation or that a steady

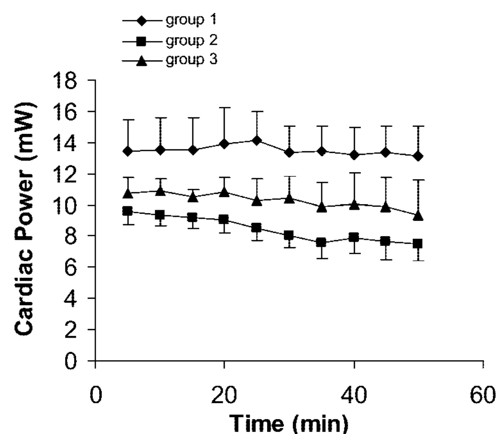

Fig. 1. Performance of hearts: cardiac power. Values are means ± SE.

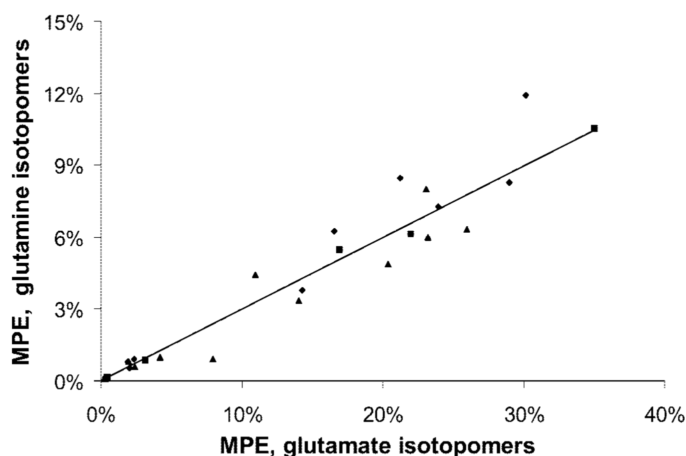

Fig. 2. Correlation of mole percent excess (MPE) of mass isotopomers of glutamine and of glutamate. ◆, *group 1*; ■, *group 2*; ▲, *group 3*. The correlation coefficient (ρ) is 96.8%, and the slope of the line is 0.31.

state had not been reached. We therefore perfused four additional hearts under the same conditions as for *group 2* (see Table 1), except that 5 mM of L-[1-^{13}C]glutamine was present as the sole source of ^{13}C atoms (and 5 mM natural abundance sodium acetate was used instead of sodium [2-^{13}C]acetate). After perfusion for 60 min, there was a strong signal from [1-^{13}C]glutamine, yet no detectable [1-^{13}C]glutamate in the heart, implying that the deamidation of glutamine occurred much more slowly than the amidation of glutamate, if at all.

Table 2. *Ratios of concentrations of glutamine to glutamate and relative rates of anaplerosis*

Group	[Glutamine]/[Glutamate]	Relative Anaplerosis, *y*
1	94.10 ± 26.68	4.9 ± 4.1
2	53.41 ± 15.47*	9.8 ± 4.3
3	96.90 ± 44.70	16.2 ± 0.2†

Each value is the mean ± SD (%) of the number of experiments shown in Table 1. *$P < 0.05$ compared with *groups 1* and *3*. †$P < 0.05$ compared with *group 1*.

Relative anaplerosis. The relative areas of the multiplets corresponding to carbons C3 and C4 of glutamate were used to estimate relative anaplerosis. A spectrum from a heart in *group 1* illustrates the chemical shift differences of carbons C1 to C4 of glutamate and glutamine and the (methyl) carbon C2 of acetate (Fig. 3). Not unexpectedly, relative anaplerosis was more than threefold higher in the propionate-perfused hearts and twofold higher in the pyruvate-perfused hearts compared with hearts perfused with glucose and insulin (Table 2). This finding is consistent with earlier observations showing enhanced rates of anaplerosis in retrogradely perfused rat hearts (7).

DISCUSSION

The main finding of this study is the direct production of glutamine from glutamate in isolated, working rat heart, suggesting the existence of a substrate cycle, which can have important implications for the regulation of cardiac metabolism. Under the present experi-

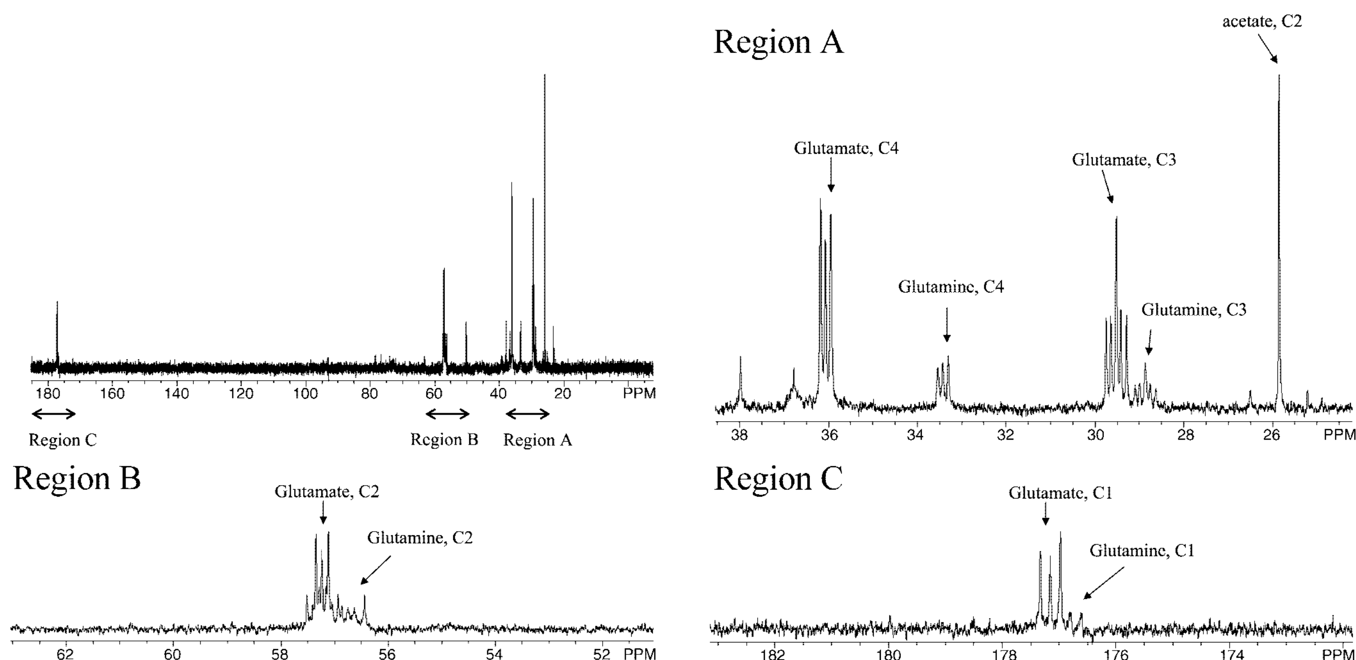

Fig. 3. ^{13}C nuclear magnetic resonance (NMR) spectrum obtained from a single heart in *group 1*. Three regions (designated *A, B,* and *C*) of the spectrum are magnified and displayed separately. Multiplets formed by carbons at positions C1, C2, C3, and C4 of glutamate and glutamine are shown, as well as carbon C2 of acetate. Chemical shifts are relative to 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt, or DSS. NMR parameters are given in the text.

mental conditions, we could not detect the closing of the cycle, i.e., the deamidation of glutamine. However, an exhaustive study of the conditions that might facilitate the deamidation of glutamine has not been attempted. The important new observation remains that, in heart, carbon arising from the citric acid cycle finds its way into glutamine.

Although the increase in sensitivity of metabolic regulation afforded by the glutamate-glutamine substrate cycle has been discussed for the case of skeletal muscle (16), no one has previously entertained the possibility of this substrate cycle in heart tissue. Allosteric regulation of glutamine synthetase and of glutaminase would allow the pool of glutamine to serve as a reservoir for the citric acid cycle, transferring mass into or out of the pool of glutamate, from which carbons are transferred to the intermediates of the citric acid cycle. Newsholme (15) has demonstrated that substrate cycles increase the sensitivity of control of mass transfer between the two metabolic pools.

The provision of [2-^{13}C]acetate to the perfused heart and comparison of the subsequent distribution of isotopic labels on glutamate and on glutamine provided evidence for the metabolic activity of glutamine synthetase. Experimental protocols were chosen to maximize the relative rate of anaplerosis (ratio of anaplerosis to citric acid cycle flux), which, we propose, would increase the turnover of glutamine. We did not observe a correlation between relative anaplerosis and maintenance of the glutamine pool in the isolated heart, although the provision of pyruvate exogenously decreased the relative concentration of glutamine to glutamate. The existence of exchange between glutamate and glutamine in working heart will need to be considered in future kinetic models of the citric acid cycle in isolated working rat heart, because it provides a source of anaplerosis into the citric acid cycle. We did not detect anaplerosis from glutamine under the experimental conditions examined in the present studies. Methods for measuring anaplerosis may consider glutamine as a possible source of ^{13}C-labeled as well as unlabeled anaplerosis (the analysis for which is described in Ref. 4). In addition, the time course of enrichment of the [^{13}C]glutamine pool under conditions of steady state will need to be determined. The major limitation of the present study is the qualitative nature of the observations and our inability to demonstrate glutaminase activity in the intact, isolated, perfused heart.

The significance of the postulated glutamate-glutamine substrate cycle is still difficult to define. We propose that, under appropriate conditions, higher workloads may stimulate the turnover of glutamine, thereby providing finer control of the increased metabolic rate and a greater degree of adaptability for the survival of the animal. We suggest that, during periods in which the rate of metabolism is relatively constant, the substrate cycle will have low activity. Under conditions that deplete the metabolites of the citric acid cycle (such as ischemia), glutamine may provide a reservoir of carbon atoms that can be used to replenish the depleted pools. Indeed, some workers found that the presence of glutamine in the perfusate of the working, perfused rat heart protects against postischemic reduction in cardiac output and in concentrations of the citric acid cycle intermediates (8). It remains to be seen whether inhibitors of either glutamine synthetase or glutaminase are specific enough to define the role of the glutamate-glutamine substrate cycle in protection of cardiac metabolism during periods of change in the cellular environment.

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DISCLOSURES

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