Volume 212, number 1, 58–62 FEB 04408 February 1987

Carbon flux through citric acid cycle pathways in perfused heart by ^{13}C NMR spectroscopy

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Received 4 November 1986

Mathematical models of the TCA cycle derived previously for ^{14}C tracer studies have been extended to ^{13}C NMR to measure the ^{13}C fractional enrichment of [2-^{13}C]acetyl-CoA entering the cycle and the relative activities of the oxidative versus anaplerotic pathways. The analysis is based upon the steady-state enrichment of ^{13}C into the glutamate carbons. Hearts perfused with [2-^{13}C]acetate show low but significant activity of the anaplerotic pathways. Activation of two different anaplerotic pathways is demonstrated by addition of unlabeled propionate or pyruvate to hearts perfused with [2-^{13}C]acetate. In each case, the amount of [2-^{13}C]acetate being oxidized and the relative carbon flux through anaplerotic versus oxidative pathways are evaluated.

^{13}-C NMR; Glutamate isotopomer; (Perfused heart)

1. INTRODUCTION

^{13}C NMR spectroscopy is a powerful tool for measuring the contribution of competing metabolic pathways in intact cells and tissues [1–5]. It offers the distinct advantage over conventional ^{14}C labeling methods in that the ^{13}C enrichment at each carbon position in a molecule may be quantitated in intact tissue. A previous heart perfusion study showed that the doublet/total intensity ratio in the glutamate C4 resonance provides a direct measure of the fraction of acetyl-CoA derived from a particular ^{13}C enriched oxidizable substrate [6]. This measurement assumes that the only carbon entering the TCA cycle is derived from the oxidizable substrate via acetyl-CoA. Although this may be a reasonable assumption under some steady-state perfusion conditions, the technique is not appropriate under conditions where TCA cycle intermediates are being replenished via anaplerotic reactions. Such reactions include pyruvate carboxylation [7] and the pathways leading to net oxidation of amino acids or odd numbered fatty acids [8–10]. Previous studies of acetate oxidation in the rat heart have shown that about 90% of the acetyl-CoA entering the TCA cycle is derived from this substrate [11–13]. Other studies have noted that the flux through anaplerotic pathways in glucose perfused hearts is about 8% of the citrate synthase flux [14,15]. These approaches require measurement of the specific activity of [6-^{14}C]citrate, the TCA cycle flux, and acetate utilization. We have applied models of the TCA cycle derived for ^{14}C tracer studies [16–18] to the analysis of heart spectra obtained under steady-state ^{13}C enrichment conditions. We report here a method for the determination of carbon flux through the oxidative (net

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

oxidation of the acetyl group to CO_2 and water) and anaplerotic pathways in intact, perfused rat hearts or heart extracts by ^{13}C NMR spectroscopy.

2. MATERIALS AND METHODS

Standard reagents were obtained from Sigma. [2-^{13}C]Acetate enriched to 99% was obtained from KOR Isotopes. Male Sprague-Dawley rats (300–350 g) were anesthetized with intraperitoneal pentobarbital and heparin (200 units) was injected intravenously about 1 min before the heart was excised. The aorta was cannulated and the heart was perfused at 70 cm H_2O with standard Krebs-Henseleit medium bubbled continuously with a 95% O_2/5% CO_2 gas mixture. The heart and perfusate temperatures were maintained at 37°C and the O_2 tension at the level of the aorta cannula was >550 mmHg. The heart rate was monitored throughout the NMR experiment. ^{13}C NMR spectra were obtained at 75.45 MHz as reported [6]. Intact heart spectra were collected in 5 min blocks (200 scans) until no further changes in the spectra were apparent. A final steady-state spectrum (1200 scans) was then collected and the experimental parameters of interest were derived from this spectrum as outlined in the text. The glutamate C3/C4 ratio was corrected for small differences in nuclear Overhauser enhancement as follows: (C3/C4) corrected = 0.90 (C3/C4) observed.

3. RESULTS AND DISCUSSION

3.1. *Effects of activation of an anaplerotic pathway on the ^{13}C NMR spectrum*

A high resolution, proton decoupled ^{13}C NMR spectrum of a rat heart perfused to steady-state with [2-^{13}C]acetate as its sole substrate is shown in the lower panel of fig.1. This spectrum is similar to that reported [6] of a guinea pig heart perfused with [3-^{13}C]lactate, showing high levels of glutamate and a single resonance from

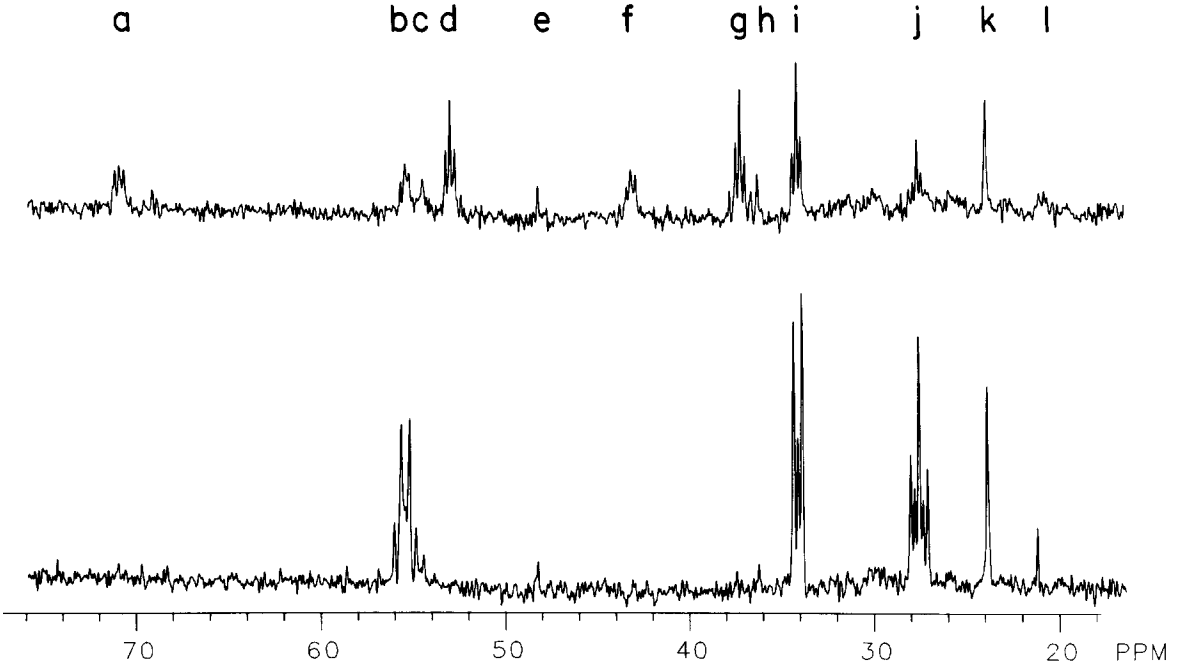

Fig.1. ^{13}C NMR spectra of a Langendorff perfused rat heart. After perfusion of the heart with 2.5 mM [2-^{13}C]acetate for 30 min, the spectrum in the lower panel was obtained. Propionate (2.0 mM) was then added to the perfusate and after 40 min of re-equilibration, the spectrum in the upper panel was obtained. Peak assignments are: a, malate C2; b, glutamate C2; c, trimethylamino resonance of acetyl carnitine plus carnitine (natural abundance); d, aspartate C2; e, taurine (natural abundance); f, malate C3; g, aspartate C3; h, taurine (natural abundance); i, glutamate C4; j, glutamate C3; k, acetate C2; l, methyl resonance of acetyl carnitine (^{13}C enriched). A prominent resonance at 136 ppm (not shown) was also detected and assigned to fumarate C2,C3.

[2-^{13}C]acetyl carnitine. [2-^{13}C]Acetate was selected for these studies because this substrate insures that ^{13}C is entering the TCA cycle only via [2-^{13}C]acetyl-CoA. Acetate perfusions also correspond to relatively low activity of the anaplerotic pathways in the heart [11]. The upper panel of fig.1 shows a ^{13}C NMR spectrum of the same heart perfused to steady-state with [2-^{13}C]acetate plus 2 mM unlabeled propionate. Propionate was used as a co-substrate since it enters the TCA cycle only via an anaplerotic pathway, i.e., as succinyl-CoA.

There are two major differences between these steady-state spectra. First, as expected [8], the entry of unlabeled propionate into the TCA cycle significantly increases the concentration of all 4 carbon TCA cycle intermediates (as reflected by the malate, aspartate and fumarate resonances) and decreases the steady-state levels of the 5 carbon intermediate pools (as reflected by glutamate). Second, the ^{13}C enrichment at the glutamate C2 or C3 positions relative to the glutamate C4 position has decreased in the acetate plus propionate perfusion, and the multiplet patterns due to spin-spin coupling have changed (see the glutamate C4 resonance in the upper and lower panels, for example).

3.2. *Model of the TCA cycle*

Sophisticated mathematical models of the TCA cycle have been developed and validated to interpret ^{14}C tracer results [16–18]. These models may be readily extended to the analysis of ^{13}C spectra of perfused hearts under steady-state conditions. The variables in the model developed for analysis of ^{13}C spectra (fig.2) include: F_c, the fraction of acetyl-CoA taking part in the citrate synthase reaction that is [2-^{13}C]acetyl-CoA; a, the flux through all other TCA cycle intermediate pools (collectively called the anaplerotic reactions) expressed as a fraction of the citrate synthase flux, c; and the sizes of the 4, 5, and 6 carbon TCA cycle intermediate pools. It was assumed that c and a would not change during the accumulation of a steady-state NMR spectrum, and, since the concentration of TCA cycle intermediates are not changing in steady-state, the pathways for removal of carbon skeletons (i.e., via the malic enzyme [8]) are as active as the pathways which replenish the intermediates.

The model predicts that the metabolic

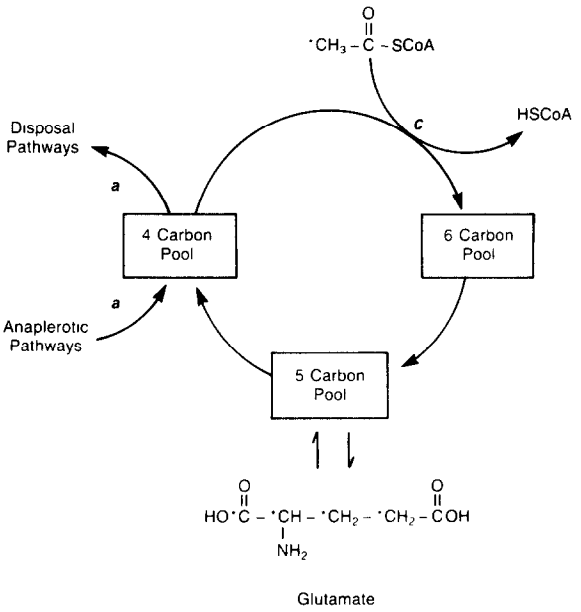

Fig.2. Model of the citric acid cycle for calculation of glutamate isotopomers. Flux through the anaplerotic pathways, a, is defined in our model as a fraction of the flux through citrate synthase, c.

parameters of interest may be derived quite simply from two experimental parameters:

$$\text{glutamate C3/C4} = 1/(2y + 1) \quad (1)$$

$$\text{glutamate C4 doublet/total} = F_c/(2y + 1) \quad (2)$$

where $y = a/c$. In comparison to ^{14}C tracer studies [16–18], our F_c is equivalent to the specific activity of [2-^{14}C]acetate after dilution with endogenous sources of acetate. The 4, 5 and 6 carbon intermediate pool sizes influence the time required to reach steady-state labeling [5] but not the final glutamate spectrum.

If the anaplerotic reactions are completely inactive ($a/c = 0$), ^{13}C enrichment at glutamate C2, C3, and C4 is equal, and the doublet/total ratio at C4 directly reports F_c [2,6]. If anaplerotic reactions are active ($a/c > 0$), the 4 carbon TCA cycle pool is diluted with ^{12}C and this is reflected in a diminution of the total ^{13}C enrichment of glutamate at C2 and C3 relative to C4. The C2 and C3 positions are always equally labeled in steady-state because of scrambling at these positions in the succinate and fumarate pools.

Table 1

Fractional enrichment and flux data from ^{13}C NMR spectra of perfused hearts

Substrates	NMR observation		F_c, fractional ^{13}C enrichment of acetyl-CoA entering the TCA cycle	a/c, flux through the combined anaplerotic reactions, relative to flux through citrate synthase
	C3/C4	C4 doublet/total		
2.5 mM [2-^{13}C]acetate (n = 4)	0.83 ± 0.02	0.81 ± 0.01	0.97 ± 0.02	0.10 ± 0.01
2.5 mM [2-^{13}C]acetate 1.0 mM propionate (n = 4)	0.57 ± 0.07	0.53 ± 0.04	0.94 ± 0.13	0.39 ± 0.10
2.5 mM [2-^{13}C]acetate 2.5 mM pyruvate (n = 4)	0.74 ± 0.03	0.57 ± 0.04	0.77 ± 0.07	0.17 ± 0.03
2.5 mM [2-^{13}C]acetate 5.0 mM glucose 5 U/l insulin (n = 7)	0.74 ± 0.08	0.67 ± 0.15	0.90 ± 0.17	0.18 ± 0.03

These results (mean ± SD) were derived from ^{13}C NMR spectra of the extracts of freeze clamped hearts as described in the text

3.3. *Analysis of ^{13}C spectra to obtain quantitative metabolic data*

We have measured the glutamate C3/C4 ratio and the glutamate C4 doublet/total ratio in hearts perfused with four different combinations of substrates. The source of ^{13}C was in each case [2-^{13}C]acetate. The results are presented in table 1. The data show that 90% or more of the acetyl-CoA entering the TCA cycle is derived from [2-^{13}C]acetate in hearts perfused with acetate alone or with acetate plus propionate or glucose and insulin. Unlabeled pyruvate, however, effectively competes with [2-^{13}C]acetate for entry into the cycle via pyruvate dehydrogenase and consequently F_c drops to 0.77. There is significant flow of unlabeled carbons through the TCA cycle pools (a/c = 0.10) when [2-^{13}C]acetate is presented as the sole exogenous substrate. Addition of unlabeled pyruvate or unlabeled propionate increases the anaplerotic flux through two completely different pathways (i.e., pyruvate enters via pyruvate carboxylase to form oxaloacetate while propionate carbons enter as succinyl-CoA), while the fraction of acetyl-CoA derived from acetate is reduced only by pyruvate. Glucose, even in the presence of insulin does not complete effectively with acetate for oxidation.

3.4. *Analysis of ^{13}C spectra in the intact heart*

These principles may be applied to the analysis of ^{13}C spectra obtained in the intact heart (fig.1). After correction for small T_1 differences, the C3/C4 ratio was 0.80 under [2-^{13}C]acetate perfusion and 0.48 under [2-^{13}C]acetate plus propionate perfusion. Thus, a/c = 0.12 in the presence of acetate and increased to 0.54 when propionate (2 mM) was added to the perfusate. The fraction of acetyl-CoA derived from acetate was about 90% under both conditions. The simplicity of this technique offers the possibility of measuring flux through multiple pathways in a single, intact heart under a variety of physiological, hormonal, and workload conditions.

ACKNOWLEDGEMENTS

This study was performed during the tenure of a Clinician-Scientist Award of the American Heart Association to C.R.M. with funds contributed in part by the Texas affiliate. The study was supported in part by grant AT-584 from the Robert A. Welch Foundation (A.D.S.). We thank Drs Ray L. Nunnally and James T. Willerson for their continued support of these experiments.

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