In vivo carbon-13 nuclear magnetic resonance studies of heart metabolism

(13C labeling/glycogen metabolism/glutamate/tricarboxylic acid cycle)

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ABSTRACT  Guinea pig heart metabolism was studied in vivo by 13C NMR at 20.18 MHz. High-quality proton-decoupled 13C NMR spectra with excellent signal-to-noise ratios and resolution could be obtained in 6 min. Natural-abundance spectra showed resonances that could be assigned to fatty acids, but glycogen was not seen. During intravenous infusion of D-[1-13C]glucose and insulin, the time course of myocardial glycogen synthesis was followed serially for up to 4 hr. Anoxia resulted in degradation of the labeled glycogen within 6 min and appearance of 13C label in lactic acid. Infusion of sodium [2-13C]acetate resulted in incorporation of label into C-4, C-2, and C-3 positions of glutamate and glutamine, reflecting "scrambling" of the label expected from tricarboxylic acid cycle activity. Examination of the 31P NMR spectrum of the guinea pig heart in vivo demonstrated no change in the high-energy phosphates during the time periods of the 13C NMR experiments. Our studies indicate that 13C NMR is a unique nondestructive tool for the study of heart metabolism in vivo.

A full understanding of the regulation of metabolic processes in vivo must rely on studies that allow continuous, nondestructive monitoring of metabolic fluxes under different physiological or pathophysiological conditions. Over the past 8 years, it has been shown by several workers that 31P NMR is a useful, nondestructive tool that can monitor the energy status and the pH of cellular suspensions and perfused organs (for reviews see refs. 1-6). Noninvasive 31P NMR studies have been performed in vivo on brain (7, 8), liver (9), kidney (10), and human forearms (11). Recently, Grove et al. (12) have also studied high-energy phosphate metabolism in rat heart in vivo. Despite its usefulness as a noninvasive technique, however, 31P NMR is limited to the detection of ATP, phosphocreatine, inorganic phosphate, phosphodiesters, and sugar phosphates. By contrast, 13C NMR spectroscopy is potentially more versatile as a tool for metabolic studies in vivo. The utility of 13C NMR for the investigation and quantitation of metabolic fluxes in a variety of cells, tissues, and perfused organs is well established (1, 13-20). However, there has only been one report indicating the feasibility of 13C NMR studies in vivo (21). Alger et al. obtained natural-abundance 13C NMR spectra of fatty acids in rat head, rat abdomen, rat hind leg, and human arm. In a preliminary experiment, these workers also demonstrated the incorporation of 13C label from D-[1-13C]glucose into liver glycogen in vivo (21).

In the studies presented here, we have examined the natural-abundance 13C NMR spectra of guinea pig heart in vivo and observed the incorporation of 13C label into myocardial glycogen and amino acids. Furthermore, the temporal resolution achieved in the 13C NMR spectra made it possible to investigate metabolic fluxes.

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MATERIALS AND METHODS

Chemicals. Sodium [2-13C]acetate and D-[1-13C]glucose (both 90 atom % 13C) were from Merck Sharp & Dohme. All other chemicals were reagent grade.

Animals. Adult Hartley strain guinea pigs (Charles River Breeding Laboratories), weighing between 600 and 750 g, were maintained on water and chow ad lib until the day of study.

Preparation of in Vivo Hearts. Animals were anesthetized with sodium pentobarbital (25 mg/kg intraperitoneally) and mechanically ventilated on room air through a tracheal cannula. An intravenous polyethylene (PE-50) catheter was placed in the jugular vein and maintained patent with an isotonic saline infusion. The animals were placed on a platform warmed to 40°C through a circulating water coil. The thorax was opened through a mid-sternal incision, and the pericardial sac was removed. The heart was positioned within the receiver rf coil.

NMR Measurements. 31P and 13C NMR spectra of heart in vivo were obtained at 32.5 MHz and at 20.18 MHz, respectively, on a TMR-32/200 spectrometer (Oxford Research Systems, Oxford, England). Quadrature phase detection and CYCLOPS pulse phase cycling were employed (22). Spectra were obtained without the use of a field/frequency lock. The magnetic field was shimmed by using the 1H NMR resonance from the heart. The linewidth of the H2O signal was generally in the range of 25–35 Hz.

For the 31P experiments, a five-turn solenoidal coil made of 1-mm high-purity copper wire (A. D. MacKay Metals, Darien, CT) was placed around the heart. The 90° pulse width was 10 μsec, determined on a sample of 100 mM inorganic phosphate or 20 mM inorganic phosphate in 150 mM KCl. Spectra were usually acquired with 3-kHz spectral width, pulse angles of 30–80°, and a recycle time of 1 sec.

13C experiments in vivo were performed with a six-turn solenoidal receiver coil mounted inside a one-turn saddle-shaped decoupling coil made of 1-mm copper wire. The latter coil was tuned to 60.29 MHz. Bilevel proton decoupling was employed, with ca. 0.5–0.8 W during the delay and 3–4 W during data acquisition. In some experiments gated proton decoupling was used, with the decoupler on only during data acquisition. No adverse effects of the proton decoupling on the animal were observed. The design and performance of the above coil combination for in vivo 13C NMR studies of heart and other organs will be described in more detail elsewhere. The 90° pulse width was 12 μsec, determined on a sample of 1 M D-[1-13C]glucose in 150 mM KCl. 13C NMR spectra were acquired with a spectral width of 10 kHz, an acquisition time of 0.1 sec, pulse angles of 60–70°, and recycle times in the range of 0.3–2 sec.

Experimental Protocol for 13C Experiments in Vivo. After several natural-abundance 13C NMR spectra of the heart in vivo had been obtained, serial spectra were obtained during the following experiments: (i) D-[1-13C]Glucose and insulin infusion;
0.5 or 0.75 g of D-[1-13C]glucose mixed with 50 units of regular insulin in a volume of 6 ml (pH 7.4) was infused through the jugular catheter at a rate of 0.05 or 0.1 ml/min. (ii) Sodium [2-13C]acetate infusion: 1.2 g of sodium [2-13C]acetate in 5 ml (pH 7.4) was infused intravenously over 60 min.

13C NMR Spectra of Heart Extracts. At the termination of an experiment, the heart was rapidly excised, rinsed with saline, and freeze-clamped between aluminum plates at liquid nitrogen temperature. Perchloric acid extracts of hearts were obtained by homogenizing the frozen tissue in 3–4 vol of 7% perchloric acid in water in a precooled 55-ml tube. The homogenate was centrifuged for 15 min. The pellet was reextracted with 7% perchloric acid and centrifuged, and the supernatants were combined. Salts were precipitated by dropwise addition of 3 M potassium carbonate under rapid stirring to achieve a pH of 6.0. The precipitate was removed by centrifugation and the supernatant was lyophilized. All extraction steps were carried out at 2°C. The lyophilized extracts were dissolved in 6H2O (99.8%), and 20 µl of p-dioxane per 2 ml of extract was added to be used as internal chemical shift standard. 13C NMR spectra of extracts were obtained at 90.55 MHz on a Bruker WH-360 NMR spectrometer in 10-mm tubes, using quadrature detection and CYCLOPS pulse phase cycling. Bilevel proton decoupling was employed, with 1 W of power during the delay and 5 W of power during data acquisition. Spectra were obtained with a spectral width of 20 kHz, a pulse angle of 60°, an acquisition time of 0.41 sec, and a delay of 1.4 sec.

RESULTS

Prior to 13C NMR experiments, the 31P NMR spectrum of guinea pig heart was studied in vivo. The 31P NMR spectrum, as shown in Fig. 1, is essentially identical to that obtained from rat heart in vivo by Grove et al. (12) and contains resonances due to phosphocreatine, ATP, phosphodiester, inorganic phosphate, sugar phosphates, and 2,3-diphosphoglycerate. The spectrum was observed over periods of up to 5 hr. During this time, no change in the high-energy phosphates was detected, indicating that the animal preparation was stable during the course of our longest 13C NMR experiments.

Fig. 2 shows a 13C NMR spectrum of guinea pig heart in vivo after D-[1-13C]glucose and insulin infusion. The natural-abundance peaks I, II, and V in this spectrum are very similar to those obtained from other mammalian tissues (21) and can be assigned to fatty acids. The resonances near 170 ppm (I) and 130 ppm (II) are due to carboxyl and double-bonded carbons, respectively, and are generally 50–70 Hz wide, whereas the CH2 resonance near 30 ppm (V) is approximately 140 Hz wide, mainly due to overlapping resonances. The relatively narrow lines indicate rapid molecular motion, but we have not attempted to distinguish between triacylglycerides and phospholipids as the source of these signals. Additional natural-abundance peaks (IV a, b, and c) can be seen in Fig. 2; these resonances have not yet been identified.

13C NMR spectra were acquired continuously during the infusion of D-[1-13C]glucose and insulin, and for up to 2 hr after completion of the infusion. Each individual spectrum was the Fourier transform average of 1,200 accumulations with a recycle time of 0.3 sec and thus took 6 min for total collection. To serve as a reference for the individual spectra, Fig. 2 is a sum of seven such spectra, taken between 44 and 86 min after the start of glucose infusion. Within 6 min after the start of the infusion, we observed the resonances at 96.8 and 92.9 ppm (III b and c), which arise from the labeled C-1 carbons of the ß- and ß-amomers of D-glucose. At 15 min, the additional signal at 101 ppm (III a) was observed, due to the C-1 carbons of glycerogen (23, 24). Over the time course of the glucose infusion (1 hr), the glycerogen C-1 signal increased in intensity, as shown in Fig. 3. Upon completion of the glucose infusion, the ß- and ß-glucose signals decreased, while the C-1 glycerogen signal remained constant within the error margin.
ror of the experiment. Additional experiments were carried out with infusion times of 0.7 and 2 hr, respectively. The increase in the intensity of the C-1 glycogen signal with time during the infusion was more rapid during shorter infusions with higher infusion rates. In each case, however, the C-1 glycogen signal reached its maximal intensity approximately at the end of the glucose infusion. Within a single infusion protocol, we varied the recycle time (0.3 sec, 0.5 sec, or 1.0 sec) and compared the resultant glycogen signal. No differences in the intensities of the glycogen peaks were observed. This suggests that the C-1 glycogen resonance was not significantly saturated with the shorter recycle time.

Fig. 4 shows a 90.55-MHz $^{13}$C NMR spectrum of a perchloric acid extract of a heart that was freeze-clamped at the end of a glucose and insulin infusion. The spectrum shows resonances due to the C-1 carbons of glycogen and D-glucose. The signal at 100.6 ppm disappeared totally after the addition of 1,4-α-D-glucan glucohydrolase, confirming its assignment to the C-1 glycogen carbon. The strong signals from the α and β anomers of D-glucose in the extract suggest that there is a considerable amount of free glucose in heart muscle during glucose and insulin infusion. The glucose resonances seen in vivo result from glucose located intracellularly, in interstitial fluid, and in blood within the ventricular chambers.

The effect of anoxia on the C-1 signal of glycogen in vivo is shown in Fig. 5. A $^{13}$C NMR spectrum, accumulated in just 6 min, was obtained at the end of infusion of D-[1-$^{13}$C]glucose plus insulin (Fig. 5, spectrum a). The respirator was then turned off, and the animal was subjected to a rapidly progressive sequence of hypoxia and finally anoxia. Fig. 5, spectrum b, shows a 6-min $^{13}$C NMR spectrum that was started about 3.5 min after the respirator was turned off, and Fig. 5, spectrum c, is the difference spectrum of a and b. In Fig. 5, spectrum b, the C-1 glycogen signal has clearly disappeared, illustrating the rapid degradation of the labeled glycogen during anoxia. At the same time, a signal at 21 ppm has appeared in the difference spectrum, which is due to the C-3 of lactic acid.

The $^{13}$C NMR spectra of guinea pig heart in vitro before and after the infusion of 1.2 g of sodium [2-$^{13}$C]acetate are shown in Fig. 6. These spectra represent the Fourier-transform av-

Fig. 3. Time course of myocardial glycogen synthesis in vivo upon intravenous infusion of 0.75 g of D-[1-$^{13}$C]glucose and 50 units of insulin. a, C-1 signal of β-D-glucose at 96.8 ppm; C-1 signal of glycogen at 101 ppm. Each point was obtained from a $^{13}$C NMR spectrum acquired in 6 min. The intensities of the β-glucose signals were not corrected for partial saturation due to the short recycle time.

Fig. 4. Spectrum a is the 90.55-MHz $^{13}$C NMR spectrum of a perchloric acid extract of a guinea pig heart freeze-clamped at the end of D-[1-$^{13}$C]glucose and insulin infusion. Spectrum b is the spectrum obtained 30 min after addition of 1.5 ml of a solution of 1,4-α-D-glucan glucohydrolase (EC 3.2.1.3) (1 mg/ml) in 0.2 M sodium acetate buffer, pH 4.8, to 1.5 ml of the extract, demonstrating the hydrolysis of the labeled glycogen. Spectra were acquired with a spectral width of 20 kHz, 0.41-sec acquisition time, a 60° pulse angle, a 1-sec delay, and 3,000 transients.

Fig. 5. Effect of anoxia on the $^{13}$C-labeled glycogen. Spectrum a is a 20.18-MHz proton-decoupled $^{13}$C NMR spectrum of guinea pig heart in vivo at the end of an infusion of 0.75 g of D-[1-$^{13}$C]glucose and 50 units of insulin into the jugular vein. Spectrum b is a $^{13}$C NMR spectrum started 3.5 min after the induction of anoxia by turning off the respirator and spectrum c is the difference spectrum of b and a. Spectra were acquired as described for Fig. 2 and took 6 min each for total collection.
1606 Biochemistry: Neurohr et al.

FIG. 6. The 20.18-MHz proton-decoupled 13C NMR spectra of guinea pig heart in vivo before (b) and after (a) the infusion of 1.2 g of sodium [2-13C]acetate. Spectrum c is the difference spectrum of a and b. Spectra were acquired with a spectral width of 10 kHz, 0.1-sec acquisition time, a 60° pulse angle, and 0.8-sec recycle time. Each spectrum was obtained with 800 scans and thus required 10.7 min for total collection. Spectrum a was acquired during the first 10.7 min of sodium [1-13C]-acetate infusion into the jugular vein. A 20-Hz digital line broadening was applied to the free induction decays in a and b before Fourier transformation; for the difference spectrum (c) a 25-Hz digital filter was used. Resonances are identified as follows: I, glutamate C-2; II, citrate C-2; III, glutamate C-4; IV, glutamate C-3; V, acetate C-2.

Fig. 7 shows a 90.55-MHz 13C NMR spectrum of a perchloric acid extract of a heart freeze-clamped after 60 min of intravenous infusion of 1.2 g of sodium [2-13C]acetate. The spectrum was acquired with 3,080 transients. Resonances are identified as follows: I, glutamate C-2; II, citrate C-2; III, glutamate C-4; IV, glutamate C-3; V, acetate C-2; VI, glutamine C-4.

**DISCUSSION**

The present paper demonstrates that it is possible to obtain high-quality proton-decoupled 13C NMR spectra of heart in vivo with excellent signal-to-noise ratios and resolution in a short time of data accumulation. Prior to infusion of 13C-labeled substrates, the natural-abundance carbon spectra displayed resonances attributable to fatty acids. During infusion of either D-[1-13C]glucose or sodium [2-13C]acetate, additional peaks emerged in the spectra obtained in vivo.

Infusion of D-[1-13C]glucose (with insulin) resulted in a time-dependent accumulation of labeled D-glucose units in myocardial glycogen, which persisted throughout 2 hr of infusion. In the experiments shown in Figs. 2 and 3, the C-1 glycogen signal was essentially fully relaxed, as indicated by comparison with experiments performed using longer delays between NMR sampling pulses. In fully relaxed spectra, peak areas are directly proportional to the concentration of tissue metabolites, allowing the determination of their relative concentrations. By calibrating signal areas against a reference sample of known concentration, absolute metabolite concentrations can also be measured. However, applying NMR sampling pulses at intervals much greater than the spin-lattice relaxation times of the resonances is inefficient. Knowledge of the T1 of the substrate of interest and optimization of the pulse angle (26) allows the signal-to-noise ratio per unit time to be maximized; hence good quality spectra may be obtained in a much shorter time. These spectra can be used to monitor rapid metabolic fluxes and even to perform quantitative studies, if the resulting partial saturation of the resonances is corrected by consideration of the spin-lattice relaxation times or by comparison with fully relaxed spectra.

Recent natural-abundance 13C NMR measurements of liver glycogen have shown that despite glycogen's high molecular weight virtually all of the 13C nuclei contribute to the observed NMR spectra, presumably due to widespread intramolecular motion (24). If the same is true of heart glycogen in vivo, then the NMR spectra obtained at 20.18 MHz correspond to the total amount of D-[1-13C]glucose incorporated into glycogen.

The temporal resolution achieved in the current study allowed high-quality 13C NMR spectra of myocardial glycogen to be recorded in periods of 6 min or less (Fig. 5). This capability provides a nondestructive in vivo method to follow both the relatively slow process of glycogen accumulation and the more rapid...
processes of glycolysis or acetate incorporation into glutamate and glutamine.

Upon infusion of sodium [2-13C]acetate, incorporation of 13C label into glutamate and glutamine could be observed in vivo. On entering the tricarboxylic acid cycle as the methyl carbon of acetyl-CoA, the 13C label is introduced into the C-4 position of 2-oxoglutarate. Upon completing this first turn of the tricarboxylic acid cycle, the 13C label at C-4 of 2-oxoglutarate will move with equal probability into the C-2 and C-3 positions of this intermediate, due to the symmetry of succinate. With repeated turning of the tricarboxylic acid cycle, all the C-2, C-3, and C-4 atoms will be labeled. The ratio of the total intensity of the C-4 doublet to that of the C-4 singlet is approximately unity in the perchloric acid extract (see Fig. 7). Bailey et al. (25) reported a similar splitting pattern for C-4 glutamate in the isolated perfused rat heart. On this basis, they concluded that the acetyl-CoA pool is fully labeled. In contrast, den Hollander et al. (18) demonstrated in yeast fed acetate that full labeling of the acetyl-CoA pool initially produces a singlet for C-4 glutamate that with time converts to a doublet as the tricarboxylic acid pools are labeled. Interpreted in this light, our results indicate that the tricarboxylic acid pools, which include acetyl-CoA and glutamate pools, were only partially labeled at the time of our last measurement. Judging from spectrum c of Fig. 6, C-2, C-3, and C-4 of glutamate are approximately equally labeled, indicating rather complete scrambling through turning of the tricarboxylic acid cycle. But the total amount of labeling is only partial as judged from the glutamate C-4 triplet structure seen in the extract spectra (Fig. 7). These experiments point out that considerations of the relative labeling of the glutamate carbons in vivo, coupled with spectra of heart extracts, can give information about the degree of pool labeling and tricarboxylic acid cycle activity.

The total amino acid content of guinea pig heart muscle, after infusion of sodium [2-13C]acetate, was measured in perchloric acid extracts by using an amino acid analyzer. Glutamate and glutamine together account for 50% of the α-amino acids present in the extract. The high concentration of glutamate and a rapid equilibrium with 2-oxoglutarate probably accounts for the strong resonances of the C-2, C-3, and C-4 glutamate carbons.

Glycogen, due to its intracellular abundance, appears to be a particularly suitable substrate for 13C NMR analysis in cardiac muscle (Figs. 2 and 3). The availability of a nondestructive method for the study of glycogen synthesis (or glycolysis) will allow a reevaluation of the factors responsible for in vivo regulation of glycogen synthesis. For example, it has been difficult to demonstrate a stimulatory effect of insulin on glycogen synthesis in the perfused rat heart (27). Indeed, an increased glycogen content is found in hearts from diabetic animals (28). In contrast to the heart, insulin promotes glycogen synthesis in both liver and muscle (29, 30). The present experiments show that with 13C NMR the acute effects of insulin on glycogen synthesis in vivo can be readily measured. Likewise, the effect of physiologic or pharmacologic increases in glycogenolytic hormones (epinephrine or glucagon) or increased heart rate on glycogenolysis can be investigated because our temporal resolution allows monitoring of both slow and fast metabolic fluxes. The results demonstrate that 13C NMR spectroscopy is a unique, nondestructive tool for the study of heart metabolism in vivo. Furthermore, by combining 31P NMR and 13C NMR spectroscopy in vivo, metabolic fluxes in high-energy phosphates and myocardial glycogen under different conditions could be investigated at the same time in the same animal.