High resolution proton NMR studies of perfused rat hearts

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High resolution ^{1}H NMR spectra of perfused rat hearts have been obtained under normoxic, ischemic and hypoxic conditions. Several myocardial metabolites including taurine, carnitine, lactate and tissue glycerides are detected in the ^{1}H NMR spectra. Changes in oxygen availability induce perturbations in the levels of some metabolites, in particular, lactate. Experiments with fasted rats and with substrate-free perfusion suggest that the glycerides detected in ^{1}H spectra are metabolically mobilizable but have a slow rate of turnover. These results demonstrate that utility of ^{1}H NMR in monitoring myocardial metabolism.

^{1}H-NMR *Perfused rat heart* *Myocardial metabolism*

1. INTRODUCTION

Recent studies have solidly established high resolution nuclear magnetic resonance (NMR) as a non-invasive spectroscopic tool in studies of metabolism in intact cells. The majority of these studies have utilized ^{31}P and ^{13}C nuclei on systems such as cells in suspension [1,2] or attached to solid matrices [3], perfused organs [4–6] and whole animals [6]. Despite its relatively high sensitivity, ^{1}H nucleus has been used to a lesser extent, primarily due to problems arising from the presence of H_2O and the generally ubiquitous distribution of protons in cellular components. Under certain circumstances, these problems have been overcome to a large extent and successful applications of high resolution ^{1}H NMR on suspensions of intact cells [7,8] and subcellular organelles [9], and on whole animals [8] have been reported. Here we report ^{1}H NMR studies of perfused hearts. The results demonstrate that proton resonances are detected from several myocardial metabolites and glycerides and that ^{1}H NMR spectra are sensitive to the metabolic state of the cardiac tissue.

2. METHODS

Hearts were excised from ~300 g Sprague-Dawley rats and perfused at 38°C by the Langendorff method using Krebs-Henseleit buffer containing 11 mM glucose and equilibrated with 95% O_2/5% CO_2 gas mixture. Perfusion pressures were maintained at 68 mm Hg by gravity; typical flow rates during perfusion were 20–25 ml/min. Left ventricular pressure was continuously monitored using a catheter inserted in the left ventricle through the left atrium. Ischemic and hypoxic conditions were induced by turning off the perfusion flow, and by equilibrating the perfusion medium with 95% air/5% CO_2 gas mixture, respectively. For measurements during perfusion with substrate-free medium, hearts were first perfused under standard conditions with glucose containing media for at least 45 min; subsequently, perfusion medium was changed to Krebs-Henseleit buffer free of carbon substrates. During hypoxia and in experiments with substrate-free perfusion, hearts were electrically paced just above their basal rhythm (~ 280–300 beats/min) to maintain the heart rate constant. Perchloric acid (PCA) extracts were prepared on freeze-clamped hearts as in [10], except that the final freeze-dried powder was dissolved in 99% D_2O.

The NMR measurements were conducted at 361 MHz on a wide-bore Nicolet 360 spectrometer using an 18 mm solenoidal probe of our construction; the coil of this probe effectively consists of

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two, parallel, single turn wire solenoids. NMR spectra of perfused hearts were obtained by presaturating the water using a DANTE sequence [11] consisting of 800–1000 ~ 10° pulses and 100 μs intervals between the pulses, followed by a $(90\text{-}\tau\text{-}180\text{-}\tau)_n$ Hahn spin-echo pulse sequence. Resolution enhancement was applied on the intact heart spectra, by multiplying the free induction decay with a sine function prior to exponential filtering and Fourier transformation. Chemical shifts are reported relative to sodium 3-(trimethylsilyl)-1-propanesulfonate (TPS); for intact heart spectra, they were measured in one heart by infusing the chemical shift standard through a side-port on the input perfusion line. For extract, they were directly added into the NMR samples.

3. RESULTS AND DISCUSSION

Fig. 1 illustrates the aliphatic region of the Hahn spin-echo ^1H NMR spectra obtained from a perfused rat heart before and after the onset of global ischemia. The H_2O protons were presaturated and the large contribution of intensity coming from short-T_2 protons of the cell constituents were eliminated by the application of the Hahn spin-echo pulse sequence with 60 ms delay between the 90° and 180° pulses. No resonances were detected downfield of H_2O, in the aromatic region of the spectra. Minimum delay of approximately 30 ms was required between the 90° and 180° pulses in order to eliminate the short-T_2 contributions and discern the resonances seen in fig. 1. However, with a 30 ms delay, some resonances such as lactate are nulled due to modulation of their phases induced by spin-spin coupling; with a 60 ms delay, most doublets appear as negative peaks whereas most triplets and all of the singlets remain positive. Fig. 2 illustrates spin-echo spectra recorded from PCA extracts of freeze-clamped hearts under conditions corresponding to those of fig. 1a, 1b.

Several resonances in the extract spectra (fig.2) have been identified based on chemical shifts, spin-spin coupling, and addition of candidate compounds into the extracts; these include taurine, carnitine, phosphocreatine, creatine, glutamate, succinate, acetate, alanine, lactate and glucose. Peak identifications are given in the figure legend. In the intact heart spectra, linewidths are greater and

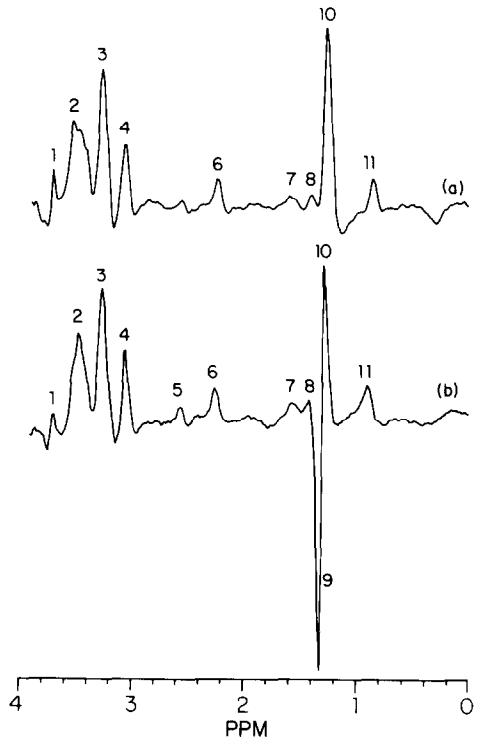

Fig. 1. 361 MHz ^1H spectra of intact rat heart under normoxic (a) and ischemic (b) conditions. Each spectrum is the sum of 64 scans recorded with Hahn spin-echo pulse sequence, 2 s repetition time, and a 60 ms delay between the 90° and 180° pulses. Resonance identifications: 1, unassigned; 2, taurine plus unidentified contribution(s); 3, carnitine -$N(Me)_3$; 4, taurine, phosphocreatine and creatine; 5, unassigned; 6, glyceride $C_\alpha H$; 7, glyceride $C_\beta H$; 8, glyceride $C_\gamma H$; 9, lactate; 10, glyceride -$(CH_2)_n$-; 11, glyceride terminal methyl.

consequently the resolution is not as good as it is in the extract spectra. In one heart, T_2 values for peaks 2 and 10 (fig.1) were measured to be 20 and 22 ms (± 10%), respectively, indicating that the intrinsic widths of these resonances are approximately a third of what is observed. At the expense of signal-to-noise ratio, some form of resolution enhancement is necessary to clearly distinguish the resonances seen in fig. 1. The 3 prominent peaks at ~ 3.4, 3.2 and 3.0 ppm (peaks 2,3 and 4, fig.1) are at the correct position for taurine, carnitine, and (taurine + creatine + phosphocreatine), respectively. These are the compounds which give rise to the intense peaks at the same chemical shifts in the extract spectra (fig.2). Taurine is present in very

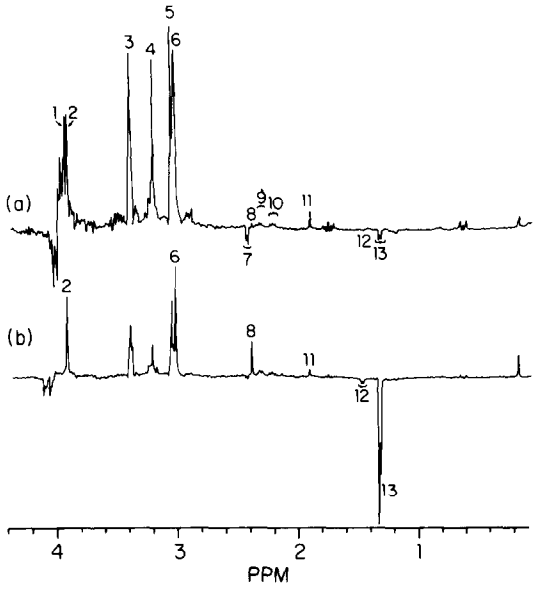

Fig. 2. 361 MHz ^{1} NMR spectra of PCA extract of freeze-clamped hearts prepared from perfused hearts under normoxic (a), and ischemic (b) conditions. Spectra were recorded with Hahn spin-echo pulse sequence using 60 ms delay between 90° and 180° pulses (38°C, pH 8.5, 200 scans, 5 s repetition time). Ischemia was global and lasted 20 min prior to freeze-clamping. Resonance identifications: 1, phosphocreatine; 2, creatine; 3, taurine; 4, carnitine -N(Me)$_3$; 5, taurine; 6, phosphocreatine plus creatine; 7, carnitine (-CH$_2$-); 8, succinate; 9,10, glutamate; 11, acetate; 12, alanine; 13, lactate. Several peaks present in spectrum (a) at ~ 3.5 ppm and ~ 3.8 ppm stem from glucose. The negative peaks at ~ 4.1 ppm in spectrum (b) arise from lactate CH.

large quantities in cardiac tissue [12]. Carnitine is not as abundant [13]; however, it has 9 equivalent -N(Me)$_3$ protons which contribute to the resonance at ~ 3.2 ppm. In addition, Hahn spin-echo spectra recorded at different τ values on intact hearts showed that peak 2 and 4 have a triplet contribution and peak 3 arose from a singlet. These observations suggest that resonances 2, 3 and 4 (fig.1) originate primarily from the protons of taurine, carnitine, creatine and phosphocreatine. Peak 2 (fig.1) clearly contains partially resolved contributions from other compounds which have not been identified. Approximately 20% of total carnitine in the myocardium exists as acid insoluble fatty acyl carnitine [13]. However, given the resonance linewidths in intact heart spectra, and the fact that the -N(Me)$_3$ moiety is well removed from the acylation site, the resonance in the ^{1}H NMR spectrum (peak 3, fig.1) probably stems from total carnitine. It should also be noted that, considering the number of protons contributing to each resonance, relative intensities of taurine and carnitine peaks in extract spectra were approximately consistent with the previously published values for the amounts of these compounds in rat hearts [12,13]. However, in the intact cell spectra relative intensities can be distorted substantially due to variations in T_2 among the different metabolites and phase-modulation induced by spin-spin couplings.

Lactate (peak 9) is assigned in the intact cell spectra on the basis of its chemical shift, the doublet nature of the resonance and its accumulation during ischemia. Succinate and alanine which are also formed during ischemia (fig.2) are not observed in fig.1b. Alanine is not formed in large quantities and there exist fairly intense, overlapping resonances in this region of the intact cell spectrum. Therefore, it is not surprising that distinct resonances from these metabolites are not detected in fig. 1b. The extent of succinate accumulation varied among the different hearts examined. It was resolved and detectable in some intact heart spectra recorded subsequent to onset of global ischemia at 2.37 ppm and not detected in others. The cause of this variation is not known.

Resonances 6, 7, 8, 10 and 11 in fig. 1 were assigned to the α, β, γ, -(CH$_2$)$_n$- and the terminal methyl protons, respectively, of fatty acid chains of glycerides and free fatty acids (FFA). These assignments are based on the characteristic chemical shifts of fatty acid protons [14] and the fact that these resonances were not observed in acid extracts. Linewidth considerations suggest that the membranes do not contribute significantly to these resonances. Detection of ^{1}H resonances primarily from the -(CH$_2$)$_n$- protons of glycerides have previously been reported in Friend leukemia cells [7], skeletal muscle [15], isolated brain cells [8] and in the whole rat brain [8]. However, only in the case of the heart, and to a lesser degree in brain cells, are the protons from the different moieties of the fatty acid chains clearly distinguishable.

Myocardial glycerides are thought to be distributed among several pools with different metabolic turnover rates as well as histologic characteristics [16,17]. In order to further define

the origin of the lipid peaks observed in the ^1H NMR specta, we examined hearts from animals fasted for 24 h, and monitored the response of hearts from animals on a normal diet to perfusion with substrate-free media. Significant alterations in the content of myocardial glycerides in response to these interventions have previously been documented [17–19]. A ~10–15-fold increase in the triglycerides was noted in cardiac tissue of rats and guinea pigs upon fasting for 24 h [18,19]; the phospholipid content was found to be stable until the third day of fasting. In 8 hearts we examined following a 24 h fasting period, 2 showed large increases in the intensity of the lipid resonances and 6 showed very low levels; typical spectra obtained from the 2 extreme responses are shown in fig. 3. Note that resonances other than those assigned to the fatty acid chains are not altered when the glycerides are either increased or diminished (fig.3). Perfusion of rat hearts with substrate-free bicarbonate buffer for ~ 30 min was shown to cause a ~ 70% decrease in the endogenous

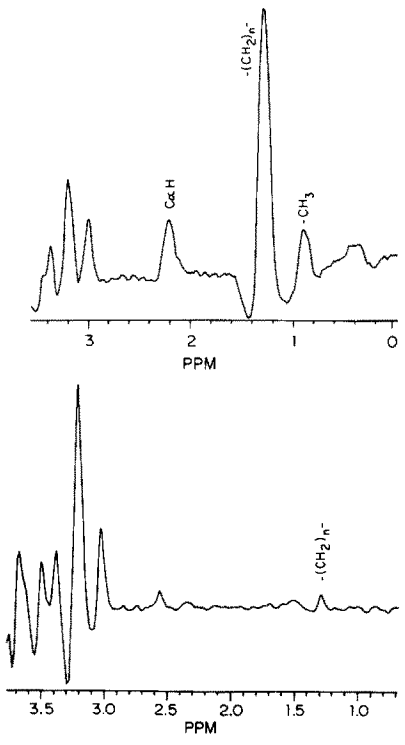

Fig. 3. 361 MHz ^1H NMR spectra of perfused rat hearts obtained after the animals were fasted for 24 h. All experimental conditions are as in fig. 1.

triglycerides, ~ 40% decrease in FFA and little or no change in the di- and monoglycerides [17]; significant additional changes were not observed when perfusion with the substrate-free media was continued for longer periods. On the basis of these and other observations, myocardial glycerides were divided into two pools, those with a high rate of turnover and those which possess a slower rate of turnover [17]. We examined the effect of substrate-free perfusion on the ^1H NMR spectra of 4 rat hearts. Alterations in the intensities of glyceride peaks were not detected 1.5 h after switching from a glucose containing medium to substrate-free medium. Our observations in conjunction with the previous reports [17–19] indicate that the resonances 6, 7, 8, 10 and 11 in fig. 1 originate from metabolically mobilizable myocardial glycerides and possible FFA but not from those which have a rapid rate of turnover. A possible assignment consistent with the previous findings in [17] would be that the NMR peaks primarily stem from mono- and diglycerides. These compounds constitute only ~ 5% of the total myocardial glycerides [17,19] and are present at a total intracellular concentration of ~ 0.7 μmol/g dry weight [19] or ~ 0.35 mM in the cytosol [20]. This is approximately one-tenth the concentration of total carnitine [13]. In the presence of uncertainties introduced by the spin-echo sequence and the resolution enhancement, the intensities of peaks 3, 6 and 11 are compatible with these concentrations. The alternative explanation is that the multiple glyceride pools which are distinguished on the basis of metabolic turnover [17] also possess distinctly different physical characteristics. Some of these glycerides including a fraction of the triglycerides are detected in the NMR spectra; these, however, do not include the large amount of triglycerides accumulated in the heart during fasting. Instead, in most cases, glyceride resonances observed in the ^1H NMR spectra undergo a drastic reduction in response to fasting. This suggests that while the circulating plasma-free fatty acids in fasting animals are extracted by the myocardium and stored as triglycerides [18,19], a certain pool of endogenous glycerides which are detected by NMR and which normally have a slow turnover are preferentially utilized.

^1H NMR spectra reflect several metabolic changes which occur in response to the state of ox-

ygenation. Accumulation of lactate and to a lesser extent of succinate is observed during ischemia. Taurine and carnitine levels showed virtually no change, consistent with earlier reports [12,13]. We have also examined hearts rendered hypoxic by equilibrating the perfusate with 95% air/5% CO_2 gas mixture. In 7 hearts examined, 4 showed three new resonances which appeared in response to hypoxia. Fig. 4 illustrates the spectrum of an hypoxic heart in which the increases in the intensity of the new resonances (peaks 1*, 2* and 3*) were largest. Examination of ^1H spectra of PCA extracts prepared from this and other hypoxic hearts showed that the new resonances do not stem from acid-soluble metabolites. These resonances remain unidentified. However, it is interesting to note that they are 0.13 ppm downfield of glyceride $C_\alpha H$, -$(CH_2)_n$-, and -CH_3 resonances and have approximately the same relative intensities as the glyceride resonances. It is possible that they arise from glycerides within a subcellular structure which experiences a slightly different magnetic field due to variations in diamagnetic susceptibility. Lactate was not detected in the ^1H NMR spectra of hypoxic hearts. This probably is due to the fact that lactate can easily traverse the cell membranes and would be washed away by the continued rapid perfusion during hypoxia. This is in agreement with earlier experiments on lactate production and accumulaton in anoxic and ischemic rat hearts [21]. If, however, hypoxia is induced under reduced coronary flow rates of the perfusate, steady-state concentrations of lactate and other compounds which can leave the cells may approach levels observable by ^1H NMR.

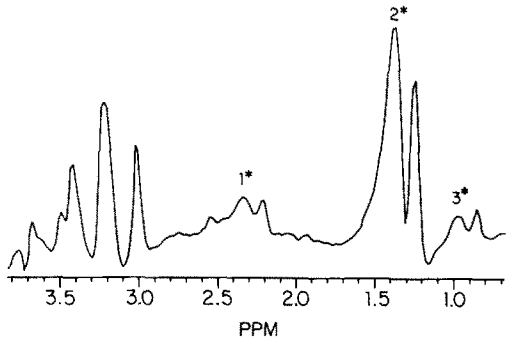

Fig. 4. 361 MHz ^1H NMR spectrum of a hypoxic perfused rat heart. The heart was initially perfused under normoxic conditions. Hypoxia was induced by equilibrating the perfusate with 95% air/5% CO_2 gas mixture.

These results demonstrate that ^1H NMR can be used to monitor aspects of myocardial metabolism which are complementary to ^{31}P NMR measurements. In addition, such ^1H NMR spectra of the cardiac muscle can in principle be obtained in whole animals using techniques by which spectra with chemical shift as well as spatial information is recorded (e.g., [22]; in this case ^1H NMR should be of great use in diagnosis of cardiac disorders.

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REFERENCES

[1] Ugurbil, K., Shulman, R.G. and Brown, T.R. (1977) in: Biological Applications of Magnetic Resonance (Shulman, R.G. ed) pp. 537–589.
[2] Shulman, R.G., Brown, T.R., Ugurbil, K., Ogawa, S., Cohen, M. and Den Hollander, J. (1979) Science 205, 160–166.
[3] Ugurbil, K., Guernsey, D.L., Brown, T.R., Glynn, P., Tobkes, N. and Edelman. I.S. (1981) Proc. Natl. Acad. Sci. USA 78, 4837–4843.
[4] Ingwall, J.S. (1982) Am. J. Physiol. 242, Heart. Circ. Physiol. H729–H744.
[5] Meyer, R., Kushmeric, M.J. and Brown, T.R. (1982) Am. J. Physiol. 242, (Cell Physiol. 11) C1–C11.
[6] Radda, G., Gadian, D.G. and Ross, B.D. (1982) Ciba. Found. Symp. 87, 36–57.
[7] Campbell, I.D. and Agois, P.F. (1982) Science 216, 1325–1327.
[8] Behar, K.L., Den Hollander, J.A., Stromski, M.E., Ogino, T., Shulman, R.G., Petroff, O.A.C. and Prichard, J.W. (1983) Proc. Natl. Acad. Sci. USA 86, 4945–4948.
[9] Ugurbil, K., Holmsen, H. and Fukami, M.H. (1983) Biochemistry, in press.
[10] Garlic, P.B., Brown, T.R., Sullivan, R. and Ugurbil, K. (1983) J. Cell. Mol. Cardiol., in press.
[11] Morriss, G.A. and Freeman, R. (1978) J. Magn. Reson. 29, 433.
[12] Crass, M.F., iii and Lombardini, J.B. (1978) Proc. Soc. Exp. Biol. Med. 157, 486–488.

[13] Idell-Wenger, J.A., Grotyohann, L.W. and Neeley, J.R. (1978) J. Biol. Chem. 253, 4310–4318.
[14] Chapman, D. and Morrison, A. (1966) J. Biol. Chem. 241, 5044–5052.
[15] Edwards, H.T., Wilkie, D.R., Dawson, M.J., Gordon, R.E. and Shaw, D.E. (1982) Lancet i, 725–731.
[16] Stein, O. and Stein, Y. (1968) J. Cell. Biol. 36, 63–77.
[17] Crass, M.F., iii, McCaskill, E.S., Shipp, J.C. and Murthy, V.K. (1971) Am. J. Physiol 220, 428–435.
[18] Wittels, B. and Bressler, R. (1964) Lab. Investigation 13, 794–799.
[19] Denton, R.M. and Randle, P.J. (1967) Biochem. J. 104, 416–422.
[20] Morgan, H.E., Regen, D.M. and Park, C.R. (1964) J. Biol. Chem. 239, 369–374.
[21] Rovetto, M.J., Whitner, J.T. and Neeley, J.R. (1973) Circ. Res. 32, 699–711.
[22] Brown, T.R., Kincaid, B.M. and Ugurbil, K. (1982) Proc. Natl. Acad. Sci. USA 79, 3523–3526.