Abnormal phosphocreatine metabolism in perfused diabetic hearts

A ^{31}P nuclear-magnetic-resonance study

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^{31}P n.m.r. analysis of control and diabetic hearts perfused for 1 h with a glucose buffer showed constant and normal levels of phosphocreatine and ATP. Supplementing the buffer with 0.5, 1.2 or 2.0 mM-palmitic acid had little or no effect on high-energy-phosphate levels in control hearts. In contrast, increases in palmitate concentration produced significant decreases in ATP in diabetic hearts, despite normal and constant levels of phosphocreatine. This ^{31}P n.m.r. study suggests a defect in phosphocreatine metabolism in the perfused diabetic heart that might be related to creatine kinase kinetics.

Phosphocreatine and ATP are decreased in the diabetic rat heart *in vivo* (Allison *et al.*, 1976). In the intact animal, diabetic ketoacidosis results in the reduction of 2,3-bisphosphoglycerate, the key modulator of O_2 binding to haemoglobin in red blood cells (Benesch & Benesch, 1967; Chanutin & Curnish, 1967). This results in a decrease in tissue oxygenation and can account for the decrease in high-energy phosphates seen in the intact animal. Despite the condition *in vivo*, there have been reports that perfusion of isolated hearts with buffer normalizes ATP (Allison *et al.*, 1976; Opie *et al.*, 1971; Rösen & Shrör, 1980). There is one report that longer-term aerobic perfusion of diabetic hearts resulted in a 20% decrease in myocardial ATP (Miller, 1979). In order to resolve this problem, we began our study by investigating the effect of perfusion duration on ATP levels in diabetic hearts. After this we studied the effect of normal and elevated levels of perfusate long-chain fatty acids on high-energy-phosphate metabolism in perfused diabetic rat hearts. It is known that the concentrations of serum non-esterified fatty acids *in vivo* increases in the diabetic (Rizza *et al.*, 1971; Murthy & Shipp, 1977), yet diabetic hearts have not been routinely perfused with elevated fatty acid levels. ^{31}P n.m.r. was used to measure sequential changes in high-energy-phosphate metabolism within individual hearts perfused with various concentrations of palmitic acid.

Experimental

Male albino Sprague–Dawley rats (Sasco, Omaha, NE, U.S.A.) weighing 450–500 g were used

in the present study. Diabetes was induced by tail-vein injection of 100 mg of streptozotocin/kg, which was dissolved in 0.1 M-citrate buffer, pH 4.5. After 48 h, blood glucose levels in excess of 13.9 mM and positive ketonuria were used as criteria for demonstrating the diabetic state.

Rats were heparinized with an intraperitoneal injection of 3000 units of sodium heparin at 20 min before anaesthetization with 5% (w/v) sodium thiamylal. Hearts were excised, arrested in 4°C heparinized perfusion medium and secured with suture to the perfusion cannula. Hearts were perfusion-washed for 10 min at constant pressure (100 cm of water) with Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 5.5 mM-glucose (Salhany *et al.*, 1979). After this equilibration period, perfusion was switched to a constant-flow (15 ml/min) system in which perfusate was continued for a duration of 1 h. The medium in this system contained, in addition to glucose, palmitic acid (0, 0.5, 1.2 or 2.0 mM) complexed to 2.5% (w/v) fatty-acid-free bovine serum albumin. The procedure for complexation of fatty acid to albumin was described previously (Crass *et al.*, 1969).

The perfusion buffer was oxygenated with O_2/CO_2 (19:1) in a device that utilizes gas-permeable silastic tubing. This system is a modification of that described elsewhere (Bergmann *et al.*, 1979; Manning *et al.*, 1980), in that perfusate flow in the 8.62 m (25 ft) of coiled silastic tubing proximal to the aorta is countercurrent to the oxygenated gas flow. Thus efficient oxygenation occurs. Furthermore, the lack of a direct liquid/gas interface inhibits foaming that can occur in proteinaceous solutions (Manning

Fig. 1. A typical series of n.m.r. spectra from a diabetic rat heart

Major resonance peaks are identified. The heart was perfused at constant flow for 1 h with a recirculating Krebs–Henseleit bicarbonate buffer containing 5.5 mM-glucose and 2.5% fatty-acid-free bovine serum albumin. Each spectra represents a 10 min scan starting at the top for baseline control levels. Time intervals for collection of n.m.r. spectra from top to bottom are 2–12, 13–23, 24–34, 35–45, 46–56 min of recirculation perfusion. Values in

et al., 1980). Perfusate temperature was also calibrated to provide 37°C at the entrance to the aorta.

Physiological indices of hearts were monitored on a Gilson ICM-5H polygraph recorder. Heart rate and aortic pulse pressure were derived from a Statham P23Db transducer connected to the perfusion inflow line.

After perfusion was switched to the recirculation mode, cannulated hearts were inserted into the magnetic field of a Nicolet NTC 150 spectrometer (Nicolet Instrument Corp., Mountain View, CA, U.S.A.). The probe diameter was 20 mm. Spectra were obtained from 420 transients of 78° pulses delivered at 1.024 s intervals. In all cases, line broadening was 3 Hz. For other details, see Pieper *et al.* (1980). After 2 min of recirculation perfusion, the baseline n.m.r. spectrum was collected. Sequential n.m.r. spectra were collected throughout the length of perfusion. A period of 1 min was allowed to elapse between scans. Thus the last scan in the series was terminated after 56 min of perfusion. This allowed 4 min to remove cannulated beating hearts from the magnetic field and the n.m.r. tube for freeze-clamping tissue. ATP and phosphocreatine were analysed (Lamprecht & Stein, 1965; Lamprecht & Trautschold, 1965) as well as additional biochemical studies not described in detail in the present study.

Relative intensities of n.m.r. peaks were integrated as described previously (Pieper *et al.*, 1980) and were normalized as a percentage of each individual heart's baseline value. Intracellular pH was determined as discussed previously (Busby *et al.*, 1978; Pieper *et al.*, 1980). Statistical significance ($P < 0.05$) was determined by analysis of variance or by paired *t* and unpaired *t* analysis where appropriate.

Results and discussion

The major discovery of the present study was an abnormality in high-energy-phosphate metabolism in the perfused diabetic rat heart. Fig. 1 shows a qualitative example of the sequential n.m.r. spectra of a diabetic heart that was perfused for 1 h with Krebs–Henseleit bicarbonate buffer, with glucose as the sole exogenous substrate. The expected n.m.r. peaks are shown along with the extracellular and intracellular components of P_i (Pieper *et al.*, 1980). Viability of both control (spectra not shown) and diabetic hearts was demonstrated by the constant levels for phosphocreatine and ATP throughout the

parentheses indicate integrated areas for that peak in absolute units. $P_i(ex)$ and $P_i(in)$ represent extracellular and intracellular components of inorganic phosphate respectively. Abbreviation used: PCr, phosphocreatine.

duration of the experiment. Unlike control hearts (spectra not shown), diabetic hearts perfused with 2.0 mM-palmitate plus glucose showed marked declines in intracellular ATP levels, with phosphocreatine remaining virtually at normal levels (Fig. 2).

Quantitative analysis of n.m.r. peaks for all control and diabetic hearts perfused with 0, 0.5, 1.2 and 2.0 mM-palmitate are summarized in Table 1. In control hearts, duration of perfusion and the concentration of perfusate palmitate had little or no effect on levels of high-energy phosphates. In only one instance was there a slight decrease in ATP in the last scanning interval in those control hearts perfused with 2.0 mM-palmitate.

Results for diabetic hearts shown in the lower portion of Table 1 show a markedly different phenomenon. There were significant decreases in ATP with duration of perfusion. The decline in ATP occurred during normal (0.5 mM) and elevated (1.2 and 2.0 mM) non-esterified-fatty-acid perfusions. The largest loss in ATP occurred in the presence of 2.0 mM-palmitate. Furthermore, the onset of ATP decrease appeared earlier at higher palmitate concentrations. The approximate decrease in ATP observed in the diabetic hearts is physiologically significant in that hearts have been demonstrated to fail with only a 20% decrease in ATP (Neely & Morgan, 1974). Unexpectedly, we observed no significant decreases in phosphocreatine throughout the duration of perfusion in each diabetic group. Particularly noteworthy is the complete absence of change in phosphocreatine content in those diabetic hearts perfused at 2.0 mM-palmitate despite the *simultaneous and significant* loss of ATP.

^{31}P -n.m.r. spectra were also analysed for the intramyocardial pH of control and diabetic hearts. No significant change in pH occurred during perfusion of both control and diabetic hearts. Intracellular pH at the beginning and end of fatty-acid-free perfusion was $7.07 \pm 0.01 \rightarrow 7.06 \pm 0.01$ (control) and $7.06 \pm 0.01 \rightarrow 7.08 \pm 0.01$ (diabetic). Diabetic hearts perfused with 0.5, 1.2 and 2.0 mM-palmitate had values ranging from $7.10 \pm 0.01 \rightarrow 7.08 \pm 0.01$, $7.08 \pm 0.01 \rightarrow 7.08 \pm 0.01$ and $7.10 \pm 0.02 \rightarrow 7.10 \pm 0.02$ respectively.

The ^{31}P n.m.r. data on phosphocreatine and ATP was confirmed from direct chemical analysis from frozen tissue extracts of the same hearts used in the n.m.r. studies. Briefly, ATP levels in diabetic hearts were decreased from corresponding levels in control hearts. In hearts perfused with 2.0 mM-palmitate, ATP levels were $11.83 \pm 1.52 \mu\text{mol/g}$ dry wt. (control) and $7.66 \pm 1.43 \mu\text{mol/g}$ dry wt. (diabetic). Corresponding levels of phosphocreatine were unchanged (21.47 ± 1.74 and $20.78 \pm 1.93 \mu\text{mol/g}$ dry wt. respectively).

The n.m.r. and chemical-analysis data together demonstrate a remarkably unique phenomenon. In

Fig. 2. A typical series of n.m.r. spectra from a diabetic rat heart, perfused under conditions detailed in the legend to Fig. 1 except that the perfusate was supplemented with 2.0 mM-palmitate

Scaling for this Figure is different from that for Fig. 1. This does not affect subsequent calculations, as they are normalized as a percentage of the baseline spectra. Abbreviation used: PCr, phosphocreatine.

Table 1. Summary of ^{31}P -n.m.r. data for control and diabetic perfused rat hearts

Values are expressed as the mean percentage $\pm$ s.e.m. Baseline values were collected at 2–12 min into recirculation perfusion and arbitrarily set at 100%. Statistical significance (paired t analysis versus baseline values for that group): * $P < 0.05$; † $P < 0.025$; ‡ $P < 0.01$; § $P < 0.005$.

Condition	n	[Palmitate] (mm)	Time (min)	[PCr] (% of baseline control value)					[ATP] (% of baseline control)				
				13–23	24–34	35–45	45–56	...	13–23	14–24	35–45	46–56	
Control	4	0.0	...	104.5 $\pm$ 4.0	96.6 $\pm$ 2.8	99.4 $\pm$ 4.4	100.5 $\pm$ 2.9		102.4 $\pm$ 2.3	101.7 $\pm$ 4.1	103.4 $\pm$ 0.9	107.8 $\pm$ 4.5	
	6	0.5		92.6 $\pm$ 3.3	97.1 $\pm$ 3.5	94.6 $\pm$ 3.1	93.7 $\pm$ 3.5		99.6 $\pm$ 3.2	102.2 $\pm$ 5.6	97.2 $\pm$ 3.8	95.4 $\pm$ 5.3	
	5	1.2		108.5 $\pm$ 2.8	107.7 $\pm$ 2.2	104.1 $\pm$ 3.2	101.5 $\pm$ 2.5		94.1 $\pm$ 8.2	94.5 $\pm$ 3.6	97.5 $\pm$ 1.2	95.9 $\pm$ 4.5	
	6	2.0		103.1 $\pm$ 1.0	101.8 $\pm$ 1.3	99.8 $\pm$ 1.9	102.3 $\pm$ 1.3		98.8 $\pm$ 1.8	104.3 $\pm$ 6.4	99.5 $\pm$ 4.4	93.4* $\pm$ 1.6	
	6	0.0		106.2 $\pm$ 2.9	108.6 $\pm$ 6.4	106.1 $\pm$ 3.0	100.4 $\pm$ 4.1		91.4 $\pm$ 5.7	95.6 $\pm$ 4.2	95.5 $\pm$ 6.5	93.9 $\pm$ 7.3	
Diabetic	6	0.5		104.9 $\pm$ 6.5	102.1 $\pm$ 6.1	101.3 $\pm$ 3.5	103.6 $\pm$ 3.7		92.4 $\pm$ 5.5	89.7 $\pm$ 5.8	86.4† $\pm$ 4.9	79.9§ $\pm$ 4.1	
	7	1.2		98.9 $\pm$ 1.1	102.8 $\pm$ 3.5	98.7 $\pm$ 2.4	101.6 $\pm$ 3.0		93.1 $\pm$ 5.0	88.9* $\pm$ 6.6	87.4* $\pm$ 4.2	85.3† $\pm$ 4.2	
	7	2.0		106.7 $\pm$ 2.8	103.6 $\pm$ 5.2	103.0 $\pm$ 4.5	100.6 $\pm$ 3.5		94.7 $\pm$ 6.0	86.3* $\pm$ 4.2	76.8† $\pm$ 5.6	76.0 $\pm$ 7.1	

other forms of myocardial stress, such as O_2 -deficient states (regional ischaemia, global ischaemia, hypoxia, anoxia or excess catecholamines), a significant decrease in phosphocreatine is always seen before any significant decline in ATP (Salhany *et al.*, 1979; Pieper *et al.*, 1980; Scheuer & Stezoski, 1968; Gauduel *et al.*, 1979; Dube' *et al.*, 1982). Our demonstration of decreased ATP, but constant phosphocreatine, in the diabetic heart tends to suggest a disruption in normal phosphocreatine metabolism. A potential defect in creatine phosphokinase is indicated, since it is the only known enzyme that utilizes phosphocreatine (Lohman, 1934). The maintenance of a normal steady-state concentration of phosphocreatine implies that flux towards phosphocreatine breakdown is inhibited under these conditions. It is not known at this time whether this is a result of direct effect on the kinetics of creatine phosphokinase. Absence of a change in intracellular pH as demonstrated by n.m.r. is evidence that a change in pH is not a factor in any potential alteration in the apparent K_{eq} for creatine kinase. We have observed, however, a good correlation between the level of ATP loss and the elevation of long-chain fatty acyl-coenzyme A in the same hearts (G. M. Pieter, W. T. Murray & J. M. Salhany, unpublished work). Since elevations in these CoA esters during myocardial ischaemia are known to inhibit mitochondrial adenine nucleotide translocase (Shug *et al.*, 1975), this suggests some form of interaction between translocase and creatine kinase in this diabetic model. Functional coupling (Saks *et al.*, 1975) as well as structural (Sharov *et al.*, 1977) of these two enzymes has been shown in the normal heart. Further support for this hypothesis is the perfusion of hearts with atractyloside, an irreversible inhibitor of the translocase (Klingenberg *et al.*, 1975). ^{31}P -n.m.r. data showed a significant 25% decrease in ATP with no change in phosphocreatine (G. M. Pieper, J. M. Salhany & S. T. Wu, unpublished work).

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