Non-invasive investigation of myocardial energetics in cardiac disease using ^{31}P magnetic resonance spectroscopy

Mark A. Peterzan*, Andrew J. M. Lewis, Stefan Neubauer, Oliver J. Rider

University of Oxford Centre for Clinical Magnetic Resonance Research (OCMR), Radcliffe Department of Medicine, University of Oxford, Oxford, UK

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Correspondence to: Professor Oliver J. Rider. University of Oxford Centre for Clinical Magnetic Resonance Research (OCMR), Level 0, John Radcliffe Hospital, Oxford OX3 9DU, UK. Email: oliver.rider@cardiov.ox.ac.uk.

Abstract: Cardiac metabolism and function are intrinsically linked. High-energy phosphates occupy a central and obligate position in cardiac metabolism, coupling oxygen and substrate fuel delivery to the myocardium with external work. This insight underlies the widespread clinical use of ischaemia testing. However, other deficits in high-energy phosphate metabolism (not secondary to supply-demand mismatch of oxygen and substrate fuels) may also be documented, and are of particular interest when found in the context of structural heart disease. This review introduces the scope of deficits in high-energy phosphate metabolism that may be observed in the myocardium, how to assess for them, and how they might be interpreted.

Keywords: Energetics; magnetic resonance spectroscopy; cardiac magnetic resonance (CMR)

Introduction

Cardiac metabolism and function are intrinsically linked. High-energy phosphates occupy a central and obligate position in cardiac metabolism, coupling oxygen and substrate fuel delivery to the myocardium with contractile work. This insight underlies the widespread use of stress echocardiography to document inducible wall motion deficits and interpret these as ischaemia: the ischaemic cascade begins with a metabolic perturbation, climbs through diastolic dysfunction, inducible regional wall motion abnormality, and culminates in electrocardiographic changes and symptoms. However, inducible ischaemia is not the only metabolic problem that may or may not be phenotyped in the heart with impaired contractile reserve. Other deficits in high-energy phosphate metabolism (not secondary to supply-demand mismatch of oxygen and substrate fuels) can be documented, and are of particular interest when found in the context of structural heart disease.

This review introduces the scope of deficits in high-energy phosphate metabolism that may be observed in the myocardium, how to assess for them, and how they might be interpreted.

The significance of high-energy phosphates in myocardial work and energy homeostasis

A high-energy phosphate molecule carries a phosphoryl bond ($\sim$P) with a large energy of hydrolysis. Reactions that add water and split off the phosphoryl group release inorganic phosphate (P_i) and lots of energy (J/mol). These exergonic reactions (negative free energy, or delta G)

*, ORCID: 0000-0002-1793-3128.

are coupled in living cells to molecular motors which require energy to do work. The most important molecular motors in the heart include sarcolemmal Na^+-K^+-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase, and myosin-ATPase. Each carries a thermodynamic requirement for a work cycle to proceed spontaneously (i.e., the sum of the negative delta G of ~P hydrolysis and the positive delta G of the work-performing enzyme must be less than zero). Without ~P availability, the cell cannot do work or maintain ionic homeostasis as ~P hydrolysis is obligate for both. This is also referred to as contractile coupling:

Cardioventilatory coupling → Oxygen delivery → ~P → Sarcomere shortening → External work.

The predominant high-energy phosphate is ATP, which has two ~P bonds. ATP hydrolysis results in ADP + P_i. While ADP could be hydrolysed to AMP, and AMP to adenosine, in practice cells try to avoid this as adenosine is lost easily from cells by diffusion and ATP is resource-intensive to synthesise *de novo* (requiring nine ATPs) (1). Cells also try to maintain very high [ATP]/[ADP][AMP] ratios (phosphorylation potentials) near work-performing enzymes. A first reason for this is that the free energy of ATP hydrolysis is proportional to the [ATP]/[ADP][P_i] ratio.

$$\Delta G_{ATP} = \Delta G^0_{ATP} - RT \ln\left\{[ATP]/[ADP][P_i]\right\} \quad [1]$$

A second reason is that high cytosolic [ADP] antagonises myosin-actin cross-bridge cycling, and raises the left ventricular end-diastolic pressure-volume slope (2,3). Third, [AMP] is a marker of metabolic stress, and directly activates AMP-activated protein kinase (AMPK). AMPK is an evolutionarily-conserved enzyme that acts as a sensor of metabolic stress and responds by activating ATP-supplying catabolic pathways and suppressing ATP-requiring anabolic pathways (4,5). Fourth, P_i is a direct stimulant of glycogenolysis and anaerobic glycolysis (at the level of phosphofructokinase). Glycogen is the only carbohydrate store in cardiomyocytes and is metabolically costly to synthesise and to maintain (attracting much more water mass than lipid per unit energy stored).

In practice therefore, several mechanisms have evolved to maintain high-energy phosphate metabolic (i.e., energetic) homeostasis – high [ATP]/[ADP][AMP] ratios and low [P_i] in the cytosol. Conversely, metabolic stressors, whether they be exercise, ischaemia, pressure-overload, or glucose-deprivation, may or may not perturb this energetic signature depending on their severity, duration and the existing metabolic reserve.

The first defence against metabolic stressors a high-energy phosphate-donor (phosphagen) system, which in vertebrate muscle is the creatine kinase (CK) system (6). CK buffers ATP concentrations in cells during fluctuations in energy demand. The CK system comprises a phosphate donor (phosphocreatine, PCr), a phosphate acceptor (free creatine, Cr), and a catalytic enzyme (CK).

$$MgADP^- + PCr^{2-} + H^+ \rightleftarrows MgATP^{2-} + creatine \quad [2]$$
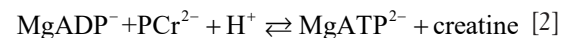
The phosphagen PCr was the first recognised high-energy phosphate (7), and is enriched in cardiac muscle leading to a PCr/ATP ratio of around 2:1 at baseline. The reversible transfer of ~P from PCr to ADP ('phosphotransfer'), established in 1935 (8), effectively triples the ~P reserve that the cardiomyocyte will willingly expend before requiring more ATP resynthesis (from fuel and oxygen) or the hydrolysis of ADP or AMP. CK enzymes are also enriched in muscle. CK isoenzymes may be cytosolic, where they exist primarily as homodimers (CK-MM is the predominant form in adult hearts, with CK-MB and -BB much less common), or mitochondrial, where they exist primarily as octamers (mitochondrial-CK). Cytosolic isoenzymes primarily catalyse the forward, or ATP-producing direction of the equilibrium reaction, while mitochondrial isoenzymes catalyse the PCr-producing direction.

A second line of defence is adenylate kinase, which catalyses the equilibrium 2ADP ↔ ATP + AMP. This is of minor importance in the healthy heart, where it accounts for around 10% of total phosphotransfer (9), but becomes more important in ischaemia, where it effectively allows a second ~P to be used from ATP, and in animal heart failure models, where it can take on more of total cellular phosphotransfer to compensate for any reduction in total CK activity.

Third and fourth lines of defence have already been mentioned: AMPK activation and activation of anaerobic carbohydrate catabolism.

A less immediate form of defence is cytoarchitectural remodelling to reduce the diffusion time for ATP and ADP between mitochondria and myofibrils. ATP may be delivered to (and ADP removed from) sites of use by simple diffusion (10) and facilitated diffusion (the CK system) (11). Although there is disagreement over the relative importance of these processes in cardiac muscle (12), a reduction in diffusion distances between mitochondria and myofibrils has been observed in M-CK/mitochondrial-CK double knockout mouse myocardium (13). The promotion of "direct channelling" of adenine nucleotides between organelles
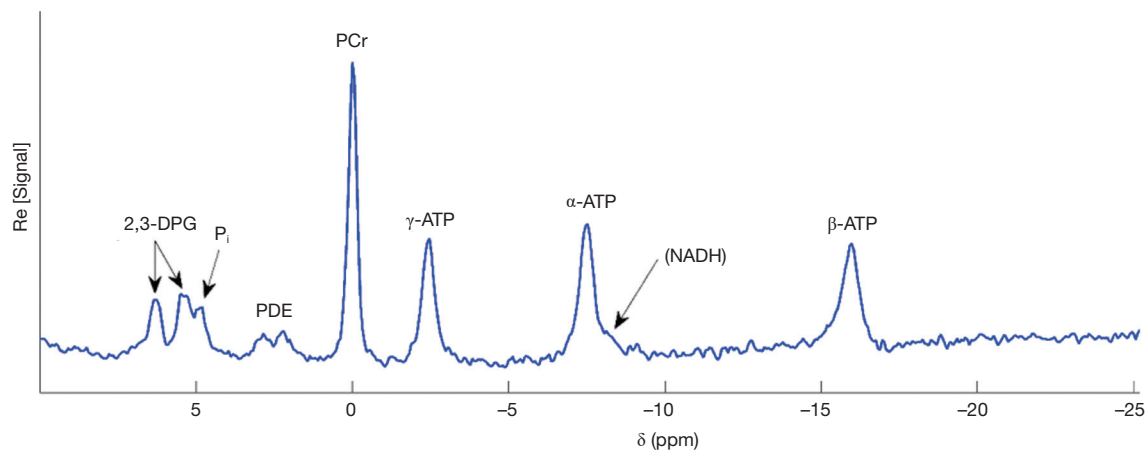
Figure 1 Cardiac ^{31}P-MRS spectrum obtained from a healthy volunteer at 7T (15). A signal in the spectrum is referred to as a resonance. Resonances with chemical shifts more positive than a reference resonance are said to lie downfield of the reference. 2,3-DPG, 2,3-diphosphoglycerate in red blood cells; PDE, phosphodiesters; P$_i$, inorganic phosphate; PCr, phosphocreatine; γ-ATP, β-ATP, α-ATP, adenosine triphosphate ~P bond in order of descending distance of the ~P bond from adenosine; NADH, nicotinamide adenine dinucleotide.

in the setting of reduced CK activity may underlie the preservation of cardiac function under moderate workloads in these hearts [reviewed (14)].

In summary, ATP occupies a central and obligate position in cardioventilatory coupling to external work. Changes in ATP homeostasis should be interpreted in the context of the prevailing metabolic stress and may indicate problems with metabolic reserve. Observed deficits in high-energy phosphate metabolism may include any or all of: reductions in [ATP][PCr]/[ADP][Cr], total CK activity, and rises in [P$_i$] and [AMP].

Techniques for interrogating myocardial high-energy phosphate metabolism

From 1927-77, biochemical analysis of rapidly flash-frozen myocardial biopsies was the only method to interrogate energetic homeostasis. This remains the gold standard for assessing total CK activity, total creatine concentration (PCr + Cr), and total adenine nucleotides (ATP + ADP + AMP). However, the lability of PCr and ATP means that PCr, ADP, and P$_i$ cannot be reliably quantified using techniques inherently destructive of the tissue under study [ATP comprises ~85% of total adenine nucleotides (2)].

^{31}Phosphorus (^{31}P)-magnetic resonance spectroscopy (MRS) is a powerful and non-invasive technique to assess cellular energetics and is complementary to biochemical analysis. All magnetic resonance techniques include two fundamental steps: firstly excitation of the nuclei of interest

using radiofrequency (RF) energy, and secondly the recording of induced current in the receiver coils of the scanner after the RF energy is switched off. The amplitude of the signal received is proportional to the number of nuclei within the interrogated volume of tissue. In MRS, the signal induced in the receiver coils by the relaxing and dephasing nuclei, or 'free induction decay', is simplified by transforming it to the frequency domain (*Figure 1*).

A ^{31}P spectrum is created by plotting signal amplitude against 'chemical shift' (δ), that is, resonant frequency (ν) relative to a reference frequency (ν$_{ref}$). The units of chemical shift are parts per million, ppm:

$$\delta\left(\text{ppm}\right) = 10^6 \times \frac{\nu - \nu_{ref}}{\nu_{ref}} \quad [3]$$

Chemical shift is field strength-independent and arises because different nuclei are shielded by nearby electrons to different extents. This shielding causes the effective field strength, and thus resonant frequency, to vary slightly between nuclei. For example, the ^{1}H spectrum of a volume of pure water should have a single peak, as all protons experience equivalent electron shielding. However, the ^{1}H spectrum of a volume of pure ethanol (CH$_3$CH$_2$OH) has three peaks, with amplitudes in a 3:2:1 ratio, corresponding to the CH$_3$-, CH$_2$-, and -OH environments.

At clinical field strengths [1.5 and 3 Tesla (T)], the resonant frequencies of ^{1}H, ^{13}C, ^{23}Na, ^{31}P are widely separated and these nuclei cannot be co-excited. However, the resonant frequencies of moeities containing ^{31}P nuclei

are much closer together—within 25 ppm of each other. Plotting the signal amplitudes against their chemical shifts relative to a designated reference nucleus (PCr) results in six visible peaks at 3T: 2,3-DPG/P_i, PDE, PCr, and three ATP peaks (*Figure 1*).

At 3T, a cardiac ^{31}P spectrum can be obtained from a 3D-localised myocardial voxel (nominal size 5.6 mL) in 11 minutes at our institution (16). In practice, 20 minutes are required if preparatory steps are included. A longer acquisition time would be required if smaller voxels or greater signal-noise-ratio was desired. Such spectra are most commonly used to estimate the PCr/ATP ratio as a key index of energetic state, but in theory could also be used to calculate absolute metabolite concentrations, intracellular [Mg] (from the shift of β-ATP relative to PCr), and pH (from the shift of P_i relative to PCr) (17). While signal-noise ratio improves linearly with field strength, these gains are only realised if increases in field strength inhomogeneity, RF energy deposition, RF thermal noise and RF energy requirements are handled acceptably (18). The potential of ^{31}P-MRS at 7T probably lies in the quantification of [P_i], a molecule that is normally obscured by the overlapping blood 2,3-DPG signal and/or too small (low in concentration) to be reliably detectable.

The key advantages of ^{31}P-MRS over biochemical techniques for the assessment of cardiac energetic state are firstly that it is non-destructive (allowing repeat assessments over time and correlation with contractile function), and secondly that it can assess the labile PCr and P_i molecules. As a result, ^{31}P-MRS has been used to study the effects of acute changes in blood flow (19-22), workload (23), substrate availability, and to assess CK activity (2,3,24,25).

The key limitation of ^{31}P-MRS is an inherently low signal-noise ratio (roughly 10^5-fold reduced compared with ^{1}H-MRS). Spectral quality can be improved by using surface coils placed as close as possible to the myocardium, although techniques to minimise contamination by skeletal wall muscle are also required. ^{31}P-MRS is relatively insensitive to metabolites at low concentrations (< ~0.5 mM), and long acquisition times. In addition, current use of many surface coils results in relative insensitivity to the posterior myocardial wall due to the greater distance from the coil. ^{31}P-MRS is therefore better suited currently to studying global rather than regional myocardial pathologies, although technical work continues to achieve whole-heart excitation.

The respective intracellular concentrations of PCr, free creatine, ATP, ADP, AMP, and P_i are on the order of 24, 16, 12, 0.04, 1, and 0.1 mM (1,26,27). In particular, the difficult

metabolites are: P_i (overlapped by 2,3-DPG), β-ADP and α-ADP (whose chemical shifts overlap those of γ-ATP and α-ATP respectively), and AMP (a phosphomonoester which lies downfield of (i.e., to the left of) P_i on a ^{31}P spectrum (*Figure 1*) and is overlapped by glucose-6-phosphate, phosphocholine and phosphoethanolamine). ^{31}P-MRS is also insensitive to non-mobile metabolites (as fixed nuclei have shorter spin-spin relaxation times (T2s), and spectral line-width is proportional to 1/T2), but in practice this is helpful as it silences signal from DNA- and macromolecule-bound nucleoside triphosphates.

In addition to acquiring snapshots of relative metabolite concentrations at single timepoints, ^{31}P-MRS can also be used to interrogate dynamic reaction kinetics. In its simplest form, this can be performed by acquiring sequential ^{31}P spectra before and after a metabolic perturbation such as exercise. Analysis of energetic and pH recovery rates can enable estimation of ATP synthesis rates (by oxidative phosphorylation and/or CK) though to date, such studies in humans have been restricted to skeletal muscle (17).

A second dynamic ^{31}P-MRS technique employs magnetisation saturation transfer. Saturation transfer techniques involve introducing a magnetic resonance label (i.e., a non-equilibrium nuclear spin magnetisation) onto a molecule of interest using selective RF pulses. If the chemical exchange is rapid enough, this magnetic label transfers to other moieties before it is relaxed by the spin-lattice (T1) mechanism. By observing this transfer, the rate constants for chemical exchange can be determined. These rate constants are unusual as they are unidirectional and first-order, even though the underlying reaction may not be.

Two broad classes of magnetisation transfer experiment include the steady state experiment, where the label is continuously introduced and signal is recorded after the system comes to a new steady state, and the temporal experiment, where the label is introduced transiently. The principles underlying these experiments have been reviewed elsewhere (28-31). The only reaction studied to date in human myocardium is the CK reaction in the ATP-producing direction, which requires continuous selective saturation of the γ-ATP resonance. Human studies date from 2002, and have employed four sequences at two centres: four-angle saturation transfer at 1.5T (32) and 7T (33), and triple repetition time saturation transfer (15,34,35) and two repetition time saturation transfer (36), both at 3T. All measure the unidirectional forward rate constant of CK, k_f, which when multiplied by substrate concentration ($k_f \times$ [PCr]) gives total (not net) unidirectional forward flux.

The reverse CK reaction could in principle be studied, as could ATP hydrolysis, but in practice the requirement to concomitantly and selectively saturate P_i remains limiting.

A third technique, ^{18}O-assisted ^{31}P-MRS (37,38) is able to infer net reaction rates by following the rate of incorporation of ^{18}O (provided from ^{18}O-labelled water) to P_i (ATP hydrolysis), to γ-ATP (ATP synthesis), and then to either PCr (CK), β-ADP and β-ATP (adenylate kinase) or glucose-6-phosphate (hexokinase). Its key disadvantage is that it requires mass spectrometry i.e., *ex vivo* tissue samples.

In summary, the above techniques collectively allow interrogation of cardiac total creatine, total CK activity, and total adenine nucleotide pool (biopsy), and of PCr, ATP, P_i, pH, CK k_f, and CK total unidirectional flux in either direction (^{31}P-MRS). The remaining moieties desirable to quantify are ADP and free creatine. ADP is usually calculated by rearranging the equation for the CK equilibrium constant, with the proviso that this extrapolates from a constant established *in vitro* for rat CK (39,40). Free creatine may be calculated by subtracting absolute [PCr] determined non-invasively (using a within-study tissue calibration) from total creatine by biopsy; it may also be non-invasively estimated by ^1H-MRS.

Deficits in high-energy phosphate metabolism that may be observed in the myocardium

Human cardiac ^{31}P-MRS studies have documented reductions in myocardial PCr/ATP ratio in established non-ischaemic cardiomyopathy (41-43), heart failure with preserved ejection fraction (44), hypertrophic cardiomyopathy (45,46), hypertensive hypertrophy with or without heart failure (47,48), severe aortic stenosis with or without heart failure (49-51), moderate aortic stenosis with preserved left ventricular systolic function (author's data, under review), severe primary mitral regurgitation in patients with an indication for operative repair [(52) and author's own data], short term high fat diet (53), obese volunteers (54,55), and patients with insulin resistance (56) or diabetes mellitus but no known heart disease (57-61). There is also a weak negative correlation with age (62-65).

A reduction in the PCr/ATP ratio is therefore an energetic signature common to numerous conditions which can predispose to heart failure, and this finding is supported by studies in animal models. Furthermore, a reduction in the PCr/ATP ratio predicts prognosis in non-ischaemic cardiomyopathy (66), and may improve with heart failure treatment (41), after aortic valve replacement (51,67), after

weight loss (68), or with trimetazidine in heart failure (69), suggesting that reduced PCr/ATP ratio is not necessarily simply an age-related phenomenon, and that energetics may be central to disease pathogenesis.

Several studies have also documented further reductions in PCr/ATP in the face of acute metabolic stress, most commonly using sustained catecholamine infusions at moderate dose (54,59,70). However, not all studies in diabetes mellitus (71) or heart failure document reduced PCr/ATP ratio at rest (72-75) or during dobutamine stress (76)—this may partly reflect a pseudo-normalisation of the resting ratio in late disease when absolute ATP concentrations reduce, and may also reflect differences in body weight, disease stage or degree of stress.

The above references are only a selection of studies performed; there is a body of animal and human work not discussed here which complements non-invasive phenotyping with biopsy assessments of total CK activity, CK isoform composition, total creatine, and/or total adenine nucleotide pool, or assesses metabolic factors upstream of ~P generation (substrate handling, mitochondrial capacity, oxidative phosphorylation). For this, the reader is referred to further reviews (77-86).

The underlying mechanism for a reduction in PCr/ATP ratio is not firmly established in all cases, but two explanations have merit in myocardial hypertrophy secondary to chronically increased work. Firstly, there may be increases in ATP consumption rate per gram of myocardium, so shifting the CK equilibrium to the right (toward ATP) in the presence of limited phosphotransfer reserve. Secondly, there may be a fall in cardiomyocyte total creatine concentration. The cell may then respond to this by shifting the equilibrium to the right, prioritising the ATP/ADP ratio at the cost of the PCr/free creatine ratio. Underlying this there may be changes in the CK equilibrium constant, which has not been assessed in human muscle or in different disease states (39,40).

Two more recently described variables in human myocardium are CK k_f and CK total unidirectional forward flux. Flux is arguably a more important variable than metabolite pool size (PCr, ATP etc) as it correlates more closely with contractile reserve (23,87-89), post-ischaemic functional recovery (37,90), and end-diastolic wall tension (2,3,24). Greater flux dampens projected beat-to-beat fluctuations in metabolite pool sizes, particularly of ATP and ADP [see (91) for assumptions implicit to *in silico* models]. Unidirectional forward flux was reduced in human hypertensive hypertrophy with failure (48), non-ischaemic

cardiomyopathy (72,92), and ischaemic cardiomyopathy (73), and this reduction in CK forward flux correlated with non-invasively estimated cardiac work (93) and carried prognostic value (72). These data are consistent with biopsy studies documenting reductions in total CK activity in human non-ischaemic cardiomyopathy (94-97) and severe aortic stenosis with or without heart failure [(98) and author's own data, under review], and with within-patient correlations of global circumferential strain by MRI, LV ejection fraction, and LV end-systolic volume index against biopsy-determined total CK velocity in patients with severe aortic stenosis (author's data, under review).

Quantifying $[P_i]$ reliably is an important goal in ^{31}P-MRS research because $[P_i]$ is required to estimate the free energy of ATP hydrolysis. It is also important kinetically, both limiting the rate of ATP hydrolysis (99) and stimulating oxidative phosphorylation (100), and at the level of substrate selection, stimulating glycogen catabolism and glycolysis. Splitting (and growth) of the P_i peak to indicate two compartments, each with different pH, is a classic indicator of acute ischaemia (101-104). In human myocardium a few groups have described the Pi/PCr ratio: in hypertrophic cardiomyopathy (75,105-107) and in non-ischaemic cardiomyopathy and healthy volunteers with and without dobutamine stress (unpublished). However, reliable signal from non-hypertrophied myocardium is only obtainable at the present time at 7T and further technical development is required.

The meaningfulness of these mostly small cross-sectional studies is somewhat paradoxical. On the one hand they afford a unique insight into ~P, a central and obligate step linking metabolic and contractile reserve. The recent observation of an increase in resting myocardial k_f and reduced responsiveness of k_f to acute dobutamine stress in obese volunteers, with reversal of the energetic phenotype after weight loss (55) raises intriguing questions about whether energetic improvement may underlie the reduction in atrial fibrillation burden also observed with weight loss (108). On the other hand, longitudinal studies of energetic phenotype controlling for covariates with known prognostic impact are lacking in a contemporary heart failure (or at-risk) cohort. Empa-Vision (NCT03332212), a longitudinal study of the effect of empaglifozin on energetic and contractile phenotype in non-ischaemic heart failure, is an example of the kind of study required to address this. The effect of cardiotoxic chemotherapy upon myocardial energetics is also relatively unstudied.

The relevance of a depressed metabolic phenotype to clinical management remains an open question, as it is not known to what extent an abnormal energetic phenotype permits or maintains the heart failure syndrome, versus simply being a bystander consequence (109). While upregulation of certain elements of the CK system can be cardioprotective [e.g., CK isozymes (110-113), total creatine (114,115)] and knockout (116) or inhibition (117) of certain elements can be detrimental, the same can be said for many parallel processes within the cardiomyocyte and do not resolve the question. Rather, they illustrate the consequence of extremes of energetic deficiency. Clinical trials of interventions that increase CK activity (118) or normalise energetic phenotypes (deliberately or not) are a required but as yet missing piece to this puzzle.

Conclusions

^{31}P-MRS affords a unique window into high-energy phosphate metabolism, which is an obligate step linking myocardial oxygen and fuel delivery with contractile work. Most work to date has focussed on the PCr/ATP ratio in various preclinical and disease states, but further work is required to understand not just whether it is depressed, but why, and to explore CK k_f and P_i more thoroughly. Together with better ^{1}H-MRS assessment of cardiac total creatine, non-invasive assessment of the amount of energy available from ATP would then become within reach. Ultimately, longitudinal studies assessing the effects of myocardial therapies on contractile phenotype, energetic phenotype, and clinical events (prognosis) will be required to assess to what extent energetic dysfunction is a bystander versus an active problem in myocardial dysfunction and heart failure.

Footnote

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