Assessing Cardiac Metabolism

A Scientific Statement From the American Heart Association

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Abstract*—**In a complex system of interrelated reactions, the heart converts chemical energy to mechanical energy. Energy transfer is achieved through coordinated activation of enzymes, ion channels, and contractile elements, as well as structural and membrane proteins. The heart's needs for energy are difficult to overestimate. At a time when the cardiovascular research community is discovering a plethora of new molecular methods to assess cardiac metabolism, the methods remain scattered in the literature. The present statement on "Assessing Cardiac Metabolism" seeks to provide a collective and curated resource on methods and models used to investigate established and emerging aspects of cardiac metabolism. Some of those methods are refinements of classic biochemical tools, whereas most others are recent additions from the powerful tools of molecular biology. The aim of this statement is to be useful to many and to do justice to a dynamic field of great complexity. (Circ Res*. 2016;118:1659-1701. DOI: 10.1161/RES.0000000000000097.)**

Key Words: AHA Scientific Statements ■ metabolic pathways ■ metabolism, cardiac ■ molecular biology ■ radionuclide imaging ■ systems biology

As a biological pump, the heart converts chemical energy into mechanical energy. This energy transfer is achieved through the coordinated activation or inactivation of enzymes, ion channels, and contractile, structural, and membrane proteins. The main goal of this statement is to describe how the many aspects of energy transfer in the heart are assessed, keeping in mind that metabolism is the transformation of a substrate to a product. The heart's energy needs are difficult to overestimate: The human heart, for example, pumps 7200 L of blood each day against an average mean arterial pressure of 100 mm Hg. In the same time period, the human heart consumes >20 g of carbohydrates (glucose and lactate) and >30 g of fat (fatty acids [FAs] and triacylglycerols [TGs]), as energy-providing substrates, whereas it uses 35 L of oxygen to support the oxidative phosphorylation of ADP to ATP.[1] In humans, the heart is responsible for ≈10% of whole-body fuel consumption. The conceptual gap between cardiac metabolism and cardiovascular health is rapidly closing, as shown by wide interest in the components of cardioprotective diets.[2]

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This statement was approved by the American Heart Association Science Advisory and Coordinating Committee on September 25, 2015, and the American Heart Association Executive Committee on October 27, 2015. A copy of the document is available at http://professional.heart.org/statements by using either "Search for Guidelines & Statements" or the "Browse by Topic" area. To purchase additional reprints, call 843-216-2533 or e-mail kelle.ramsay@wolterskluwer.com.

The Appendix is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/RES.0000000000000097/-/DC1.

The American Heart Association requests that this document be cited as follows: Taegtmeyer H, Young ME, Lopaschuk GD, Abel ED, Brunengraber H, Darley-Usmar V, Des Rosiers C, Gerszten R, Glatz JF, Griffin JL, Gropler RJ, Holzhuetter H-G, Kizer JR, Lewandowski ED, Malloy CR, Neubauer S, Peterson LR, Portman MA, Recchia FA, Van Eyk JE, Wang TJ; on behalf of the American Heart Association Council on Basic Cardiovascular Sciences. Assessing cardiac metabolism: a scientific statement from the American Heart Association. *Circ Res.* 2016;118:1659–1701. doi: 10.1161/RES.0000000000000097.

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As the cardiovascular research community is discovering a plethora of new methods for assessing cardiac metabolism, they are still scattered in the literature, many of them in the appendices of original articles. The present scientific statement seeks to provide a curated resource on methods and models used to assess basic and emerging aspects of cardiac metabolism. Some of these methods are refinements of classic tools in biochemistry, whereas others are recent additions emanating from the tools of molecular biology. An outline of the topics is shown in Table 1.

Early research on cardiac metabolism has concentrated on energy substrate metabolism, aided by techniques to cannulate the coronary sinus in vivo and to perfuse the beating heart ex vivo. Several principles have arisen from these early experiments using precise chemical or radiotracer analyses. These studies have revealed the heart as a metabolic omnivore. There are few energy-providing substrates that the heart cannot degrade in the presence of oxygen to produce ATP, when presented in sufficient quantities. In the absence of oxygen, the heart can also degrade glucose to lactate and certain amino acids to succinate through pathways coupled to substrate-level phosphorylation of ADP to ATP. A fundamental principle is that in heart muscle cells, as in any living cell, function and metabolism are inextricably linked. Modulation of cardiac metabolism, pharmacologically as well as by alteration of the metabolic milieu, to promote improved contractile efficiency of the failing heart has therefore become an attractive clinical goal.

The major fuels of respiration of the heart are fats and carbohydrates. More specifically, energy-providing substrates of the heart include TGs, long-chain FAs (LCFAs), glucose, glycogen, lactate, pyruvate, the ketone bodies acetoacetate and β-hydroxybutyrate, and a variety of amino acids, especially the branched-chain amino acids leucine, isoleucine, and valine. Each of these substrates is broken down into intermediates, entering the Krebs cycle either as acetyl coenzyme A (acetyl-CoA) or as a cycle intermediate for the stepwise production of reducing equivalents, an essential step in the process of oxidative phosphorylation. Substrate oxidation is coupled to ATP synthesis through the generation of a proton gradient across the inner mitochondrial membrane as electrons flow down the respiratory chain. The magnitude and efficiency of energy transfer in this system cannot be overestimated. The relative contribution of fat and carbohydrate to energy provision for the heart is 70% and 30%, respectively, but it varies with the physiological state.

There are other aspects of cardiac metabolism that have recently emerged as important new biological concepts: (1) The heart has the capacity to adapt to an altered physiological or metabolic environment; that is, for a given environment, the heart is able to select the most efficient substrate for ATP production. This metabolic flexibility is curtailed in the failing heart. (2) Intermediary metabolites are active participants in cell signaling, for instance, by influencing the acetylation profile of proteins that control key cellular processes.[3] (3) Intermediary metabolism is subject to circadian regulation; communication between metabolic and circadian processes orchestrates appropriate temporal organization of metabolism. (4) It is also fully recognized that metabolites are the building blocks for growth and the products of destruction of the thousands of proteins and lipid membranes comprising the cardiomyocyte. Protein quality control, autophagy, and the ubiquitin proteasome pathway each are closely intertwined with intermediary metabolism of the cardiomyocyte. (5) Beyond energy metabolism, new roles for mitochondria are increasingly being revealed. The cell's "power stations" are significant sources of signaling molecules, such as reactive oxygen species (ROS), membrane dipeptidases, cytochrome c, mitochondria-derived activation of caspases (SMAC), and Endo G, all of which, when released, impact a host of biological processes, including cell death. Mitochondria play an important role in the regulation of myocellular calcium homeostasis. Moreover, mitochondria rejuvenate themselves by undergoing fission and fusion, and fused mitochondria form an intricate intracellular tubular network. Defective mitochondria are degraded by mitophagy.

Table 1. Assessing Cardiac Metabolism: Topics

Cardiac Metabolism in Perspective
Metabolic Pathways and Networks
Systems Biology and Mathematical Modeling of Cardiac Metabolism
Measuring Metabolism
General Methodological Considerations
Rodent Strains
Sex Differences
Circadian Considerations
Age
Radiolabeled Tracers
PET/SPECT
^{31}P- and ^{1}H-MRS
^{13}C-MRS (NMR)
Stable Isotope Labeling by GCMS/LCMS
Turnover of Intracellular Macromolecules: Proteins, Glycogen, and Triacylglycerols
Directly Assessing Cardiac Metabolism In Vivo
Directly Assessing Metabolism in the Isolated Heart Ex Vivo
Metabolism of Isolated Cardiac Cells and Cell Lines
Mitochondria (Including ROS)
Proteomics and PTMs
Metabolomics
Genetic Models for Cardiac Metabolism
Nongenetic Models for Cardiac Metabolism
Metabolism of the Developing, Newborn, and Neonatal Heart
Ischemia, Reperfusion, Heart Failure, and Metabolic Modulation
Metabolism and Cardiovascular Disease: Epidemiological, Genomic, Plasma Proteomic and Metabolomic Approaches
Metabolic Signaling in Heart Disease: The Enduring Need for Quantitation

GCMS indicates gas chromatography mass spectrometry; LCMS, liquid chromatography mass spectrometry; MRS, magnetic resonance spectroscopy; NMR, nuclear magnetic resonance; PET, positron emission tomography; PTMs, posttranslational modifications; ROS, reactive oxygen species; and SPECT, single-photon emission computed tomography.

(6) Relatively subtle perturbations in metabolic fluxes can rapidly impact protein function, localization, and stability through reversible posttranslational modifications (PTMs); acetylation and O-GlcNAcylation are metabolism-derived PTMs that modulate cardiomyocyte form and function. (7) Cytokines and the immune system are increasingly recognized as regulators of cardiac metabolism. (8) Evidence for specific sequential interactions between glycolytic enzymes and between glycolytic and structural elements in the cell has accumulated. (9) In broader terms, the concept of the metabolon as a dynamic structural-functional complex that facilitates metabolite channeling is finding wider acceptance. (10) Lastly, the heart itself appears to play a pivotal role in fuel homeostasis of the body.[4,5]

The past 3 decades have also witnessed an increased understanding of the transcriptional, translational, and posttranslational control of metabolism, including the role of noncoding RNAs (eg, microRNAs or long noncoding RNAs) and selected cytokines, and they have witnessed the emergence of new conceptual paradigms. We have also gained a deepened understanding of the adaptation and maladaptation of the heart to environmental stresses, including the metabolic stresses of obesity and diabetes mellitus. Lastly, the powerful analytical tools of metabolomics and proteomics have planted cardiac metabolism firmly in the center of systems biology. As Steven McKnight noted,[3] "The vast majority of discoveries by molecular biologists over the past several decades required no attention to the metabolic state of the cell. And the sticky problems that require attention to the dynamics of metabolism were pushed aside for far too long and now loom as new challenges."

At a time when cardiovascular researchers are beginning to rethink metabolism and are in need of tools for the quantitative assessment of the dynamic process we call cellular metabolism, it is hoped that a summary of validated tools and techniques to assess cardiac metabolism will be of interest to many investigators. The aim of this article is to offer a canonical resource for both experts and newcomers alike. In addition, the statement aims to do justice to a dynamic field of great complexity.

Metabolic Pathways and Networks

With the acquisition of vast quantities of information from metabolomics, proteomics, and transcriptomics, pathway maps have evolved as a useful tool for integration of large-scale ("big") data sets. For example, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways are a collective of manually drawn maps that represent current knowledge of molecular interactions and reactive networks for metabolism,[6,7] in addition to a multitude of other cellular processes. For investigators new to intermediary metabolism, metabolic pathway mapping provides an easy and effective entry into the world of metabolic networks (Figure 1). The KEGG Atlas is available for easy access as a component of the KEGG system.[9] Note, however, that the information provided is not specific for the heart.

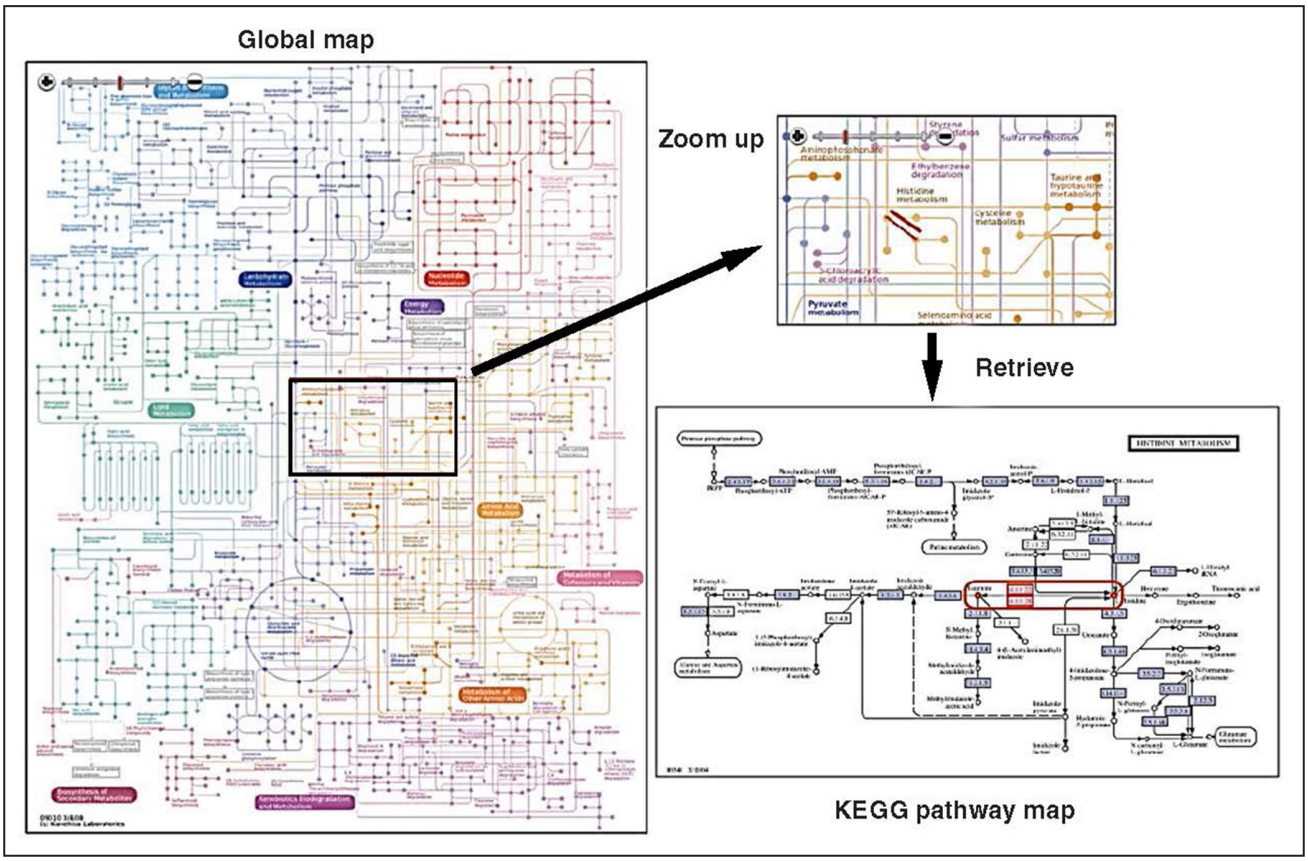

Figure 1. The main metabolic pathways for fatty acids, glucose, lactate, ketone body, and amino acids as represented in the KEGG (Kyoto Encyclopedia of Genes and Genomes) Atlas global metabolism map viewer. Reprinted from Okuda et al.[8] Copyright © 2008, The Authors (see: http://creativecommons.org/licenses/by-nc/2.0/uk/).

Given that the heart is a metabolically versatile organ capable of utilizing a large range of substrates, the main features of cardiac metabolic networks merit a brief review. For more detail, please refer to the literature listed in the Appendix.

Metabolic networks for the utilization of energy-providing substrates allow the heart to utilize distinct energy-providing substrates such as FAs, glucose, lactate, ketone bodies, and amino acids to generate ATP. Myocardial ATP stores are relatively low (eg, 300 mg in a human heart that weighs 300 g) compared with the amount of ATP required to sustain cardiac contraction (eg, 30 mg/s).[10] The contribution of each energy substrate to ATP generation is tightly regulated, and there is a significant degree of plasticity and interdependence between pathways involved in their utilization.

In the aerobic setting, >90% of the ATP produced by the heart is derived from mitochondrial oxidative phosphorylation. The remainder of ATP production occurs via substrate-level phosphorylation, which, unlike mitochondrial oxidative phosphorylation, does not require oxygen. For mitochondrial oxidative phosphorylation to occur, reducing equivalents (H^+ and electrons) are transferred from various energy-providing substrates to the mitochondria by the reduced forms of nicotinamide adenine dinucleotide (NAD-H^+) and flavin adenine dinucleotide (FADH-H^+), generated by dehydrogenase reactions that occur in the stepwise degradation of energy-providing substrates. As the main source for the production of reducing equivalents, the Krebs cycle is the central hub on which multiple metabolic pathways converge. Acetyl-CoA originating from FA, carbohydrate, ketone body, and amino acid catabolism is fully oxidized via the Krebs cycle, whereas carbon from amino acid metabolism may also enter or exit the Krebs cycle at several intermediary points.

Reducing equivalents feed into the mitochondrial electron transport chain, in which a series of redox reactions transfer electrons from donor molecules to acceptor molecules, eventually resulting in water formation through oxygen reduction. This transfer of electrons is coupled to the transfer of protons across the inner mitochondrial membrane, which creates an electrochemical proton gradient that drives ATP synthesis by ATP synthase.

The extent to which the various metabolic pathways contribute to myocardial ATP production is dependent on energetic demand, oxygen supply, the type of carbon substrate to which the heart is exposed, hormonal influences, and transcriptional, translational, and posttranslational control of the various metabolic pathways. Combined, these metabolic networks ensure that ATP production by the heart matches ATP demand on a beat-to-beat basis. The energy-providing fuels for the heart are fats, carbohydrates, ketone bodies, and certain amino acids. An outline of their metabolism is provided in the Appendix.

Practical Considerations

Should it be feasible to illustrate the transfer of information in a linear fashion from the genome ultimately to metabolic pathway flux, such a depiction may be similar to Figure 2, which illustrates the role of metabolism as a link between gene expression and function of the heart. Although each of the arrows implies a simple and predictable link from one parameter to the next, encompassing both the central dogma and the physiology of the cardiomyocyte, an appreciable number of unknown variables undoubtedly exist, ranging from gene polymorphisms, to mRNA and protein turnover, to posttranslational regulatory mechanisms (eg, allostery), and to feedback and feed-forward influences such as metabolites acting on gene expression, proteins, and enzyme activities. Extreme caution is therefore required when interpreting whether genomic, transcriptomic, and proteomic analytical outcomes translate in a predictable and reliable manner on metabolic processes. Such caution has already long been applied to a relatively distal measurable parameter included within Figure 2: Measurement of an enzymatic activity in an

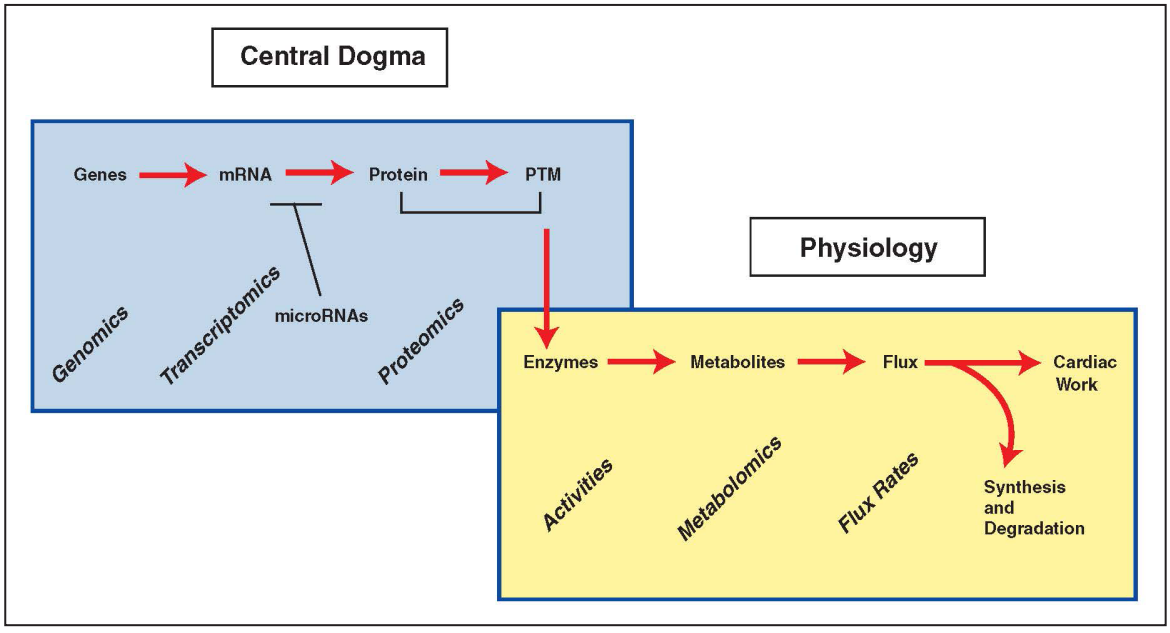

Figure 2. Hypothetical sequence from metabolic gene expression (central dogma) to energy transfer in the cardiomyocyte (physiology). The major methodologies are denoted in italics. See the Metabolic Pathways and Networks and Metabolomics sections of the text for further detail. PTM indicates posttranslational modification.

in vitro assay does not necessarily correlate with measured metabolic flux rates in an intact cell or tissue because of fluctuations in substrate, product, and allosteric regulator levels in situ, although it provides a measure of the capacity of the enzymatic conversion.

Systems Biology and Mathematical Modeling of Cardiac Metabolism

Computational methods condense experimental information from various layers of molecular organization (mRNA and protein profiles, metabolite patterns, enzyme-kinetic data) into consistent network models of cellular metabolism.[11] For cardiac metabolism, the currently practiced modeling approaches can be broadly subdivided into statistical, stoichiometric, and kinetic approaches. Multivariate statistical analyses of genome-wide "–omics" data sets, although providing only marginal insight into the regulation of metabolic networks, are useful for the identification of metabolic markers for the presence and severity of specific heart diseases.[12–14] Stoichiometric models represent basically whole-cell reaction graphs that encompass all currently known metabolites and biochemical reactions in a given cell type that can be inferred from genomic and biochemical information sources. For human cardiomyocytes, a first, although still incomplete, genome-wide reconstruction of the metabolic network is now available.[15] Combined with flux measurements based on experiments with radioactive (or preferentially ^{13}C-labeled) tracer metabolites (ie, metabolic flux rate analysis[16]) or the application of optimization principles (ie, constraint-based modeling[17]), stoichiometric models may yield estimates of stationary metabolic fluxes at varying nutrient supply or variable gene expression of metabolic enzymes or enzyme deficiencies. Kinetic models use the formalism of chemical kinetics (ordinary or partial differential equations) to relate fluxes and metabolite concentrations to the kinetic and regulatory properties of the constituting enzymatic reactions and transport processes. They allow dynamic simulations of the metabolic network and thus possess a higher predictive capacity than the aforementioned modeling types. However, because kinetic models require detailed knowledge of the underlying biochemistry, their application in metabolic research has been restricted to small subsystems, with glycolysis and mitochondrial oxidative phosphorylation as the best studied pathways in cardiomyocytes to date.[18] Much more can be expected here in the near future.

Identification of Metabolic Markers by Multivariate Statistical Analysis of High-Throughput Data

The invention of novel high-throughput technologies and simultaneous monitoring of a large spectrum of cellular macromolecules and low-weight organic molecules has promoted the development of novel methods to enable the extraction of disease-relevant information from large data sets. In particular, metabolic profiling of low-molecular-weight compounds in the plasma and urine by means of high-resolution proton nuclear magnetic resonance (^1H NMR, the standard method), liquid chromatography mass spectrometry (LCMS), and gas chromatography mass spectrometry (GCMS) is on the verge of becoming a reliable tool to assess the presence and severity of heart diseases.[19] For example, by applying supervised partial least squares discriminant analysis to orthogonal signal-corrected ^1H NMR-based metabonomics, investigators were able to define a set of plasma metabolites that enabled discrimination with ≈ 90% accuracy between normal subjects and subjects with an established coronary heart disease.[12] Orthogonal signal correction is a powerful statistical technique to remove data variation that is not correlated with the disease.[20] Currently, metabolic profiles used to identify heart disease typically consist of high concentrations of metabolites from the central metabolism that also display alterations in a wide range of other diseases. To bring the classification accuracy currently achievable with multivariate profiling methods closer to the "gold standards" set by the clinical investigation, it will be necessary to increase the sensitivity of detection methods and to develop novel modeling approaches, including the network topology and other "-omics" data. One step in this direction is the concept of reporter metabolites[21,22]; these metabolites reside in the neighborhood of reactions displaying the largest expression changes of their catalyzing enzymes. Stoichiometry-based models, including metabolic flux analysis and constraint-based modeling, as well as kinetic models, are discussed in further detail in the Appendix.

Measuring Metabolism

A common way to assess metabolism is to assess metabolite concentrations in the heart. The cross-over theorem of Chance and Williams states that when a pathway is inhibited at a specific reaction, the substrate concentration will increase and the product concentration will decrease.[23] This principle was first elucidated for the respiratory chain, for which the oxidation status of components changes rapidly in response to distinct chemical interventions, and was subsequently applied to the identification of regulatory enzymes within metabolic pathways.[24] However, the principle has not yet been applied rigorously in the analysis of contemporary metabolomic data.

Still, obtaining a snapshot measurement of specific intermediary metabolites in freeze-clamped tissue, cell organelles, or the intact heart is considered a discovery tool for metabolic regulation. Here, it is imperative to freeze the tissue instantly between aluminum blocks cooled to the temperature of liquid nitrogen.[25] Alternative methods used for the tissue preparation and metabolite extraction may be the reason for the variation in metabolic concentrations reported in the literature. A good indicator of tissue hypoxia is a lactate to pyruvate ratio >15. A list of metabolic concentrations in freeze-clamped isolated rat heart is given in Table A1 of the Appendix and may serve as a guideline for other metabolite measurements in the mammalian heart.

Metabolic flux rates assess the transfer of energy or the turnover of energy-rich intermediates. For example, although the adenylate energy charge developed by Atkinson,[26]

$$\frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}$$

takes into account that hydrolysis of ADP also yields chemical energy (besides the hydrolysis of ATP) that can be converted to useful work, the steady state concentrations of energy-rich phosphate compounds do not necessarily reflect their turnover rates. Early work in the intact heart subjected to pacing stress

in vivo demonstrated that high rates of ATP turnover are associated with only subtle differences in steady state levels of adenylate energy charge pool members.[27]

First measurements of metabolic flux rates were based on O_2 consumption measurements that rely on pressure changes to quantify the amount of O_2 consumed. The pressure changes, which derive from the cell's consumption of O_2 in a sealed container, were typically measured with a fluid manometer. As mentioned in the Appendix, the Warburg constant volume respirometer, based on a principle described by Barcroft and Haldane,[28] is an accurate but tedious method for measuring the metabolic rate of small tissue samples/homogenates and isolated mitochondria/cardiomyocytes. Today, the method has been replaced by a transient microchamber technique, which measures O_2 consumption and extracellular acidification rates in live cells at 2- to 5-minute intervals.[29]

A major limitation of metabolic flux measurements in fragmented or isolated cells is their quiescence; that is, the cells do not do any mechanical work. This limitation does not exist when metabolic flux is assessed in the intact heart, either in vivo or ex vivo. In the latter case, the isolated working rodent heart preparation affords simultaneous assessment of cardiac power development, O_2 consumption, and substrate utilization/oxidation rates.[30,31] Flux rate analyses in the intact beating heart performed with specifically labeled radioisotopic tracers (discussed in the following section) have enabled identification of all major factors that influence fluxes through specific metabolic pathways, including substrate and cofactor availability, product removal, and feedback regulation, in a highly controlled setting. Although less well controlled (in terms of variables), but more relevant to human pathophysiology, assessment of cardiac metabolic fluxes in vivo enables determination of metabolism in the presence of a neurohumoral milieu unique to the physiological/pathological status.

Furthermore, flux through metabolic pathways is not limited to energy provision for contraction It also includes ion pumps and the biosynthetic pathways of protein synthesis, ribonucleotide synthesis, lipid synthesis, and carbohydrate synthesis. These synthetic processes are, in turn, energy-requiring processes themselves, as is the degradation of macromolecules. Collectively, metabolic fluxes are an integral part of the function of the cardiomyocyte, regulated to maintain the systems of energy transfer and structure. Fluxes are responsive to changing demands on the system. A brief historical perspective on metabolic flux measurements is given in the Appendix.

Lastly, the discovery of epigenetics[32,33] has broadened the conception of intermediary metabolism in the heart, because the substrates required for all 4 forms of epigenetic changes, (ie, DNA methylation, covalent histone modifications, ATP-dependent chromatin remodeling, and noncoding RNA–mediated pathways),[34] are all derived from, or connected to, intermediary metabolism of energy-providing substrates. Several methods are available to study epigenetic changes. Among these are bisulfite sequencing to detect DNA methylation[35] and chromatin immunoprecipitation (ChIP) to study histone PTM and interactions of other proteins with DNA, such as transcription factors.[36] Lastly, noncoding RNA expression can be measured and analyzed by use of probe hybridization, RNA sequencing, and reverse transcription quantitative polymerase chain reaction.[37]

General Methodological Considerations

This subsection highlights the strengths and caveats of the different in vivo, ex vivo, and in vitro techniques for measurement of metabolic parameters, ranging from metabolites to metabolic fluxes. The aim is to draw attention to a number of distinct variables that impact virtually all metabolic assessments and therefore should be considered before studies are initiated. These include the strain of rodent model(s) (animal models), the sex of the subjects, the time of day (circadian), and the age of the subjects (whether animal models or humans) to be investigated. Each is now considered in turn.

Rodent Strains

Rodents, predominantly rats and mice, are used extensively as study models in cardiac metabolism. Specifically, for metabolic flux studies, the rodent heart remains the model of choice. Although rodent strain selection has always been part of any study design, the increased availability and use of genetically modified rodents, particularly mice, as model systems for human diseases has revolutionized the way we conduct research in general while increasing awareness about the influence of genetic background on the resulting phenotype, which encompasses the cardiac phenotype. For example, many studies have reported mouse strain variations for cardiac function,[38,39] response to β-adrenergic stimulation[40] and angiotensin II,[41] and capacity for calcium handling,[42,43] but also for susceptibility to specific pathophysiological states, such as myocardial hypertrophy[44,45] or heart failure.[46] In terms of cardiac metabolism, only a few studies have made a systematic comparison among commonly used control mouse strains. Strain-dependent variations are reported for mitochondrial enzyme activities[47] and for metabolic fluxes assessed in vivo by hyperpolarized magnetic resonance spectroscopy (MRS)[48] and in the ex vivo working heart by use of stable isotope labeling by GCMS.[49] It is noteworthy that differences are observed not only among commonly used strains but also among the mouse substrains C57Bl/6J and C57Bl/6NCrl, which are known to differ for several single-nucleotide polymorphisms. Among the mouse (sub)strains examined, 129S6/SvEvTac mouse hearts stand out by displaying a striking shift from exogenous LCFA to carbohydrate oxidation, as well as increased glycolysis and LCFA incorporation into TG, compared with 3 other (sub)strains (SJL/JCrNTac, C57BL/6J, and C57BL/6NCrl, especially C57Bl/6J hearts).[49] These differences are not readily explained by changes in transcript levels for commonly assessed metabolic genes, although there were significant variations in some myocardial transcripts among control mouse strains. Interestingly, however, 129/SvJ mice are more resistant than C57Bl/6J mice to cardiac hypertrophy development induced by pressure overload.[44,45]

Altogether, these results illustrate the importance of selecting the appropriate control (sub)strain for cardiac metabolic studies when using transgenic mice. Although strain-dependent variations in cardiac metabolic parameters can become a challenge in comparisons between studies in which different

rodent strains are used to examine the impact of a given stress challenge or treatment or in the generation of genetically modified rodents, understanding the molecular mechanisms underlying the diversity of strain-specific cardiac metabolic phenotypes may also ultimately disclose new mechanisms involved in disease development or specific targets for interventions in heart diseases.

Sex

It is becoming increasingly important in any study of cardiac metabolism to take into account sexual dimorphism. Traditionally, the vast majority of human and animal studies in the cardiovascular field have been conducted in males only or did not distinguish between the sexes. In the past decade, however, studies in the human heart have demonstrated that biological sex markedly impacts cardiac metabolism at rest,[50] in response to metabolic diseases,[51,52] and in response to metabolic medications.[53] In general, women's hearts use more FAs than men's hearts.[52] Conversely, the hearts of men use more glucose.[50] Given the inextricable links between metabolism and function and between function and outcomes, there is still a need for a better understanding of the molecular mechanisms underlying these sexual dimorphisms in cardiac metabolism. The policy of the National Institutes of Health is described in the Appendix.

Circadian Rhythms

During the planning stage, researchers consider a vast array of parameters known to influence study outcomes, as highlighted in other sections. The choice of the experimental design is often dependent on the hypothesis to be tested, information available from previously published studies, accessibility of established techniques, physiological versus pathological conditions, feasibility, expense, and perceived importance. With regard to the latter, one parameter often overlooked is the time of day. Some of the general considerations are discussed further in the Appendix.

The time of day dramatically influences biological processes, ranging from the whole organism (eg, behavior, neurohumoral factors) to the individual cell (eg, transcription, translation).[54] A process under circadian (defined as "about 1 day") regulation is metabolism.[55] Concomitant with sleep/wake and fasting/feeding cycles, marked time-of-day–dependent fluctuations in cardiac energy demand and supply occur.[56] It is therefore not surprising that cardiac metabolism changes dramatically as a function of time of day.[56,57] Moreover, in several instances, the heart appears to retain "metabolic memory," such that time-of-day–dependent fluctuations in cardiac metabolism are also observed ex vivo; oscillations in both oxidative and nonoxidative glucose, FA, and amino acid metabolism have been reported.[58–61] Similarly, energy stores (ie, glycogen, TG), metabolites, and expression of various metabolic enzymes fluctuate in the heart over the course of a day.[58–62] The magnitude of circadian regulation of metabolism is underscored by recent appreciation that metabolism is an integral component in the mammalian circadian network.[55,63] More specifically, the molecular timekeeping mechanism within individual cells, known as the circadian clock, directly modulates multiple metabolic processes in a time-of-day-dependent manner, whereas fluctuations in metabolism act in a feedback manner, thereby modulating the clock mechanism (through changes in energy charge, ROS, redox status, acetylation, O-GlcNAcylation, etc).[55,63] An added layer of complexity stems from appreciation that the responsiveness of the heart to a variety of factors known to modulate metabolism is similarly subject to circadian regulation (eg, insulin responsiveness).[64,65] In many ways, metabolism can be considered a "moving target."

Circadian regulation of metabolism has marked practical implications for the assessment of cardiac metabolism; lack of consistency of the time of day at which a parameter is measured or assessment at the "wrong" time of day can result in erroneous conclusions. For example, consider a metabolic parameter that oscillates in the rodent heart, such that the nadir (trough) is observed during the light (sleep) phase (Figure 3A and 3B). Should an experimental intervention have a positive effect on this parameter, then a greater impact might occur during the light/sleep phase (Figure 3A). Conversely, should an experimental intervention have a negative effect on this parameter, a greater impact would be predicted during the dark/awake phase (Figure 3B). It is relatively safe to assume that the majority of rodent-based experimental studies are performed during the day/light phase. Accordingly, assessment of the activator on this metabolic parameter during the light/sleep phase would likely yield a positive result. In marked contrast, assessment of the repressor on this metabolic parameter during the light/sleep phase would likely yield a null result. The latter observation would lead to the erroneous conclusion that the repressor is without significant effect on cardiac metabolism. Although only a hypothetical example, it is noteworthy that both glucose oxidation and TG synthesis, when assessed ex vivo in the mouse heart, exhibit comparable oscillations similar to those depicted in Figure 3.[60,61] Experimental interventions can also exert a wide variety of effects on time-of-day–dependent oscillations in metabolism, ranging from attenuation to augmentation, phase shift, and even initiation. For example, dietary interventions would be anticipated to influence metabolism primarily during the period of diet consumption; given that diets are consumed in a time-of-day–dependent manner, such an intervention might initiate a new metabolic rhythm. In light of the existence of rhythms in metabolic processes, the question arises regarding the best time of day during which to investigate cardiac metabolism, and this is discussed further in the Appendix.

Age

Another important aspect to take into consideration during the experimental planning stage is the effect of age on metabolism in the model(s) to be used. Aging has a significant impact on metabolism, and part of this can be explained by loss of flexibility of substrate usage.[66]

A key player affecting changes of metabolism with aging is the insulin-signaling pathway. In general, the metabolic decline that occurs during aging is related to the development of insulin resistance.[66] Hence, interventions that improve insulin sensitivity promote longevity.[66] Another important factor that changes during lifespan is mitochondrial function,

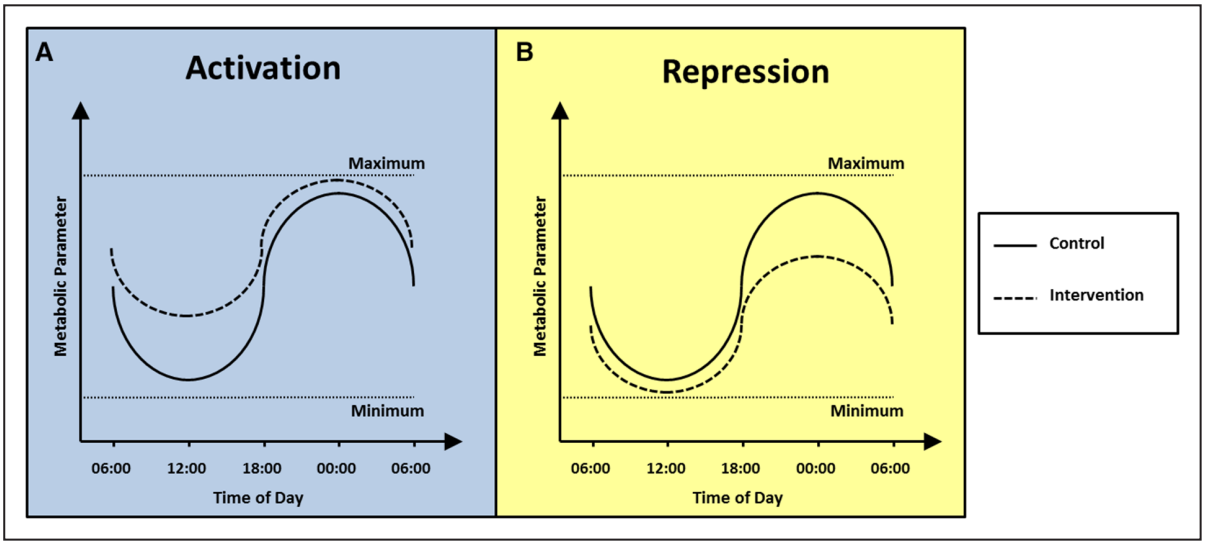

Figure 3. Hypothetical example of the impact of an activator (**A**) or repressor (**B**) on a metabolic parameter that exhibits a time-of-day-dependent oscillation. A large proportion of metabolic parameters exhibit time-of-day–dependent oscillations in the heart. As such, the time of day at which cardiac metabolism is assessed can markedly impact the results yielded and the conclusions drawn. In this hypothetical example, assessment of the metabolic parameter around 12:00 noon (light phase) would yield results consistent with the impact of the activator, whereas assessment at 12:00 midnight would not (**A**). Conversely, assessment of the metabolic parameter around 12:00 midnight (dark phase) would yield results consistent with the impact of the repressor, whereas assessment at 12:00 noon would not (**B**). Minimum and maximum indicate the physiological range for the metabolic parameter.

biogenesis, and dynamics. In the aging process, the efficiency of the electron transport chain is reduced, which results in decreased ATP generation.[67,68] Sources of mitochondrial damage include lipid accumulation from nutrient excess, ROS generation and accumulation, impaired mitochondrial dynamics, mitophagy, and mitochondrial biogenesis.[66]

In summary, to avoid confounding factors caused by the different metabolic changes that occur during health span and lifespan, the selection of a specific age for the model to be used should be considered carefully. Once the particular age of the model to be used has been selected, it needs to be kept consistent. If longitudinal studies are to be performed, appropriate controls to account for normal aging changes are necessary as well.

Radionuclide Tracers

Radioactive tracers remain the mainstays for the determination of metabolic flux rates. The principle behind the use of radioactive tracers in a biological system such as the heart is that an atom in a chemical compound is replaced by a radioactive isotope. Radioactive decay releases a high amount of energy, and although the radioactive isotope exists only in low concentrations, its presence can be detected by sensitive detectors in the form of scintillation counters or coincidence detectors. In other words, when a radioactively labeled compound is introduced into the heart, it is possible to trace the fate of the organic molecules through metabolic pathways and to quantify utilization and oxidation of the parent compound from the parent compound's specific activity (here defined as the tracer to tracee ratio). A brief historical account of the use of radioactive tracers in biology is included in the Appendix.

By strategic labeling of energy-providing substrates at various sites with either ^{3}H or ^{14}C, it is possible to collect and quantify $^{3}H_2O$ and $^{14}CO_2$ produced by the heart as a measure of energy metabolic rates. This includes the measurement of glycolysis by labeling of glucose with ^{3}H, or the mitochondrial oxidation of FA, glucose, pyruvate, ketone bodies, and amino acids with either ^{3}H- or ^{14}C (Table 2). These measurements can be made in isolated cardiac cells, heart homogenates, isolated mitochondria, or isolated perfused hearts, as well as in vivo animal models and humans. The advantages of this approach are high sensitivity (because there is no natural abundance of these isotopes) and quantitation of flux rates through the various pathways under steady state conditions. The analyses are based on the specific activity of the tracer (the tracer to tracee ratio). It is also possible to use 2 tracers (one ^{3}H and one ^{14}C) for the simultaneous assessment of flux through 2 different pathways. For instance, actual rates of glycolysis and glucose oxidation can be determined, as well as the actual contribution of carbon substrates to Krebs cycle activity. Similarly, it is possible to follow the flux of these energy substrates into various intracellular storage depots (ie, glycogen or TG) and still obtain accurate measurements of flux of exogenous energy substrates. In addition, rates of substrate uptake by the cardiomyocyte can be assessed from the accumulation of radioactively labeled tracer analogues, which are trapped as artificial intermediary metabolites. Practical considerations are discussed in the Appendix.

Table 2. Radioactive Tracers Used for the Assessment of Metabolic Pathways

Tracer Compound	Pathway Assessed
[^{32}P]	ATP turnover
[9,10-^{3}H] or [1-^{14}C]	Fatty acid oxidation
[9,10-^{3}H and 1-^{14}C]	Triacylglycerol turnover
[2-^{3}H]	Glucose uptake
[5-^{3}H]	Glycolysis
[U-^{14}C]	Glucose oxidation
[U-^{14}C]	Lactate oxidation

A limitation of using this radiolabeling approach is that only 2 metabolic pathways can be assessed at the same time, because only 1 substrate can be labeled at any given time with ^{3}H and ^{14}C. As a result, for complete assessment of energy metabolic pathways, multiple tracers are required,[69] or serial experiments are needed. This approach differs from labeling techniques with the stable isotope ^{13}C, in which multiple substrates can be measured at any given time ("Conventional ^{13}C NMR Evaluation of Cardiac Metabolism"). In short, for optimal labeling of small-molecule metabolites, knowledge of the fates of atoms and moieties in reactions is needed. Carbon fate maps and software for visualizing the maps are freely available (http://archive.is/http://cellsignaling.lanl.gov/).[70,70a]

Positron Emission Tomography/Single-Photon Emission Computed Tomography

General Principles

Radionuclide imaging with positron emission tomography (PET) or single-photon emission computed tomography (SPECT) can be used to assess myocardial metabolism in vivo. These techniques exploit the inherently high sensitivity of the detection of photons generated from different types of radioactive decay to permit in vivo 3-dimensional detection of metabolic processes and are generally applicable to patients. Because of greater resolution and quantitative capabilities, PET is the preferred approach. Another advantage of radionuclide imaging, particularly PET, is the wide range of radiotracers available to assess the metabolic fate of various plasma substrates extracted by the heart that fuel myocardial ATP production. An extensive review of the capabilities of PET and SPECT radionuclide imaging to measure myocardial metabolism has been published recently.[71] Table 3 compares the salient features of the 6 different modalities deployed for metabolic imaging in vivo described here.

One feature of PET is that radiotracers can be incorporated into a wide range of substrates or substrate analogues that participate in different biochemical pathways without altering the biochemical properties of the substrate of interest. By combining the knowledge of specific metabolic pathways with kinetic models that faithfully describe the fate of the radiotracer in the heart, an accurate interpretation of the radiotracer kinetics can be achieved. Appendix Figure A1 is the graphic representation of a 4-compartment model as an example of the steps required to quantify a metabolic process of interest using this approach. Note that in this model, uptake and clearance of the ^{11}C-labeled tracer depend on the arterial input and the metabolic fate of the tracer.[72] Conversely, transport and phosphorylation of the glucose tracer analog [^{18}F]2-deoxy-D-glucose (FDG) by heart muscle are linear with time,[73] and increased FDG retention defines a viable dysfunctional myocardium.[74] Listed in Table 4 are the metabolic processes traceable with either PET or SPECT approaches. More importantly, Table 4 highlights the breadth of metabolic processes that can be detected and quantified, the potential to measure several different processes in the same imaging session when using radiotracers with fairly short physical half-lives (eg, oxygen consumption with 1-^{11}C-acetate and FA metabolism with 1-^{11}C-palmitate), and the potential ability to detect chronic adaptations in myocardial metabolism. Conversely, shortcomings of radionuclide methods include the still relatively poor spatial resolution for SPECT, the inability to accurately characterize metabolism of substrates emanating from endogenous substrates, the general complexity of synthesis for many of the PET radiopharmaceuticals, and the exposure of human subjects to ionizing radiation.

Enhancing Translation: The Unique Challenges of Small Animal Radionuclide Imaging

To determine the relevance of the metabolic phenotypes of rodent models in various forms of human cardiovascular disease (CVD), small animal imaging systems have been developed, including multiple-pinhole PET, PET/computed tomography, SPECT, SPECT/computed tomography, and now PET/magnetic resonance (MR). However, imaging the rat and mouse heart poses unique challenges. To assess metabolism accurately, it is imperative to standardize the substrate

Table 3. Salient Features of the Modalities for Cardiac Imaging

	PET (^{11}C)	PET (^{18}F)	SPECT	NMR (^{1}H)	NMR (^{13}C)	NMR (^{31}P)
Ionizing radiation	Yes	Yes	Yes	No	No	No
High sensitivity for detection of nuclei	Yes	Yes	Yes	No	No	No
Patient applications						
Routine clinical metabolic examinations	No	Yes	Yes	No	No	No
Clinical research	Yes	Yes	Yes	Yes	No	Yes
Experimental applications						
Assess fluxes	Yes	Yes	Yes	No	Yes	Yes
Assess endogenous fuels	No	No	No	Yes	Yes	No
Assess multiple fuels	No	No	No	No	Yes	No
Assess high-energy metabolites	No	No	No	No	No	Yes

NMR indicates nuclear magnetic resonance; PET, positron emission tomography; and SPECT, single-photon emission computed tomography.

Table 4. Radiotracers Used for the Noninvasive Assessment of Cardiac Metabolism In Vivo

Radionuclide	Half-life	Compound	Present Use
SPECT			
^{123}I	13.3 h	IPPA	Fatty acid uptake, oxidation, and storage
		BMIPP	Fatty acid storage
^{99m}Tc	6.0 h	Annexin-V	Apoptosis
PET			
^{15}O	2.0 min	$^{15}O_2$	Oxygen consumption
^{11}C	20.4 min	Acetate	Oxygen consumption
		Palmitate	Fatty acid uptake, oxidation, and storage
		Glucose	Glucose uptake, glycolysis, oxidation, and glycogen turnover
		Lactate	Lactate uptake and oxidation
		KSM-01	PPARα activation
^{18}F	110 min	FDG	Glucose transport and phosphorylation
		FBEM-Cys^{40}-exedin-4	Glucagon-like peptide-1
		FTHA, FTP, FCPHA, and FTO	Fatty acid uptake and oxidation
		F7	Fatty acid uptake, oxidation and storage
		F3	PPARγ activation
			Fatty acid uptake, oxidation, and storage
		iNOS	iNOS levels
		DHE	Superoxide levels
		ICMT-11	Apoptosis
		ML-10	Apoptosis

BMIPP indicates ^{123}I-β-methyl-iodophenyl pentadecanoic acid; DHE, dihydroethidium; F3, 2-chloro-N-(4-(2-fluoroethyl)phenyl)-5-nitrobenzamide; F7, 15-(4-(2-fluoroethoxy)phenyl)pentadecanoic acid; FCPHA, *trans*-9(*RS*)-^{18}F-fluoro-3,4(*RS,RS*)-methyleneheptadecanoic acid; FDG, fluorodeoxyglucose; FTHA,14-(*R,S*)-fluoro-6-thiaheptadecanoic acid; FTO, 18-fluoro-4-thia-oleate; FTP, 16-fluoro-4-thia-palmitate; ICMT-11, *S*-1-((1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl)methyl)-5-(2(2,4-difluorophenoxymethyl)-pyrrolidine-1-sulfonyl)isatin; iNOS, inducible nitric oxide synthase; IPPA, p-^{123}I-iodophenylpentadecanoic acid; KSM-01, *p*-methoxyphenyl ureidothiobutyric derivative, a peroxisomal proliferator-activated receptor agonist; ML-10, 2-(5-fluoropentyl)-2-methylmalonic acid; PET, positron emission tomography; PPAR, peroxisome proliferator-activated receptor; and SPECT, single-photon emission computed tomography.

environment, maintain normal body temperature, deliver anesthetics that do not significantly alter metabolism, and acquire multiple serial blood samples to perform analyses for unlabeled and radiolabeled metabolites. For image acquisition and analysis, strategies are available to account for the size and rapid heart rate of the rodent heart, calculate spillover and partial volume effects, measure the arterial input function, perform calculation of various metabolic parameters with mathematical models, and validate with biochemical assays. The successful development of these techniques now permits linking observations obtained in preclinical models of disease with their human correlates.[75–78]

Clinical and Research Applications of Radionuclide Metabolic Imaging

Because of their versatility, radionuclide approaches are used extensively to detail the myocardial metabolic perturbations that characterize a wide spectrum of CVD processes. Table A2 in the Appendix lists examples for cross talk between bench and bedside. Much information can be gleaned from this table. First, the acceptance of this technology is highlighted by the routine clinical use of PET with FDG to detect dysfunctional but viable myocardium for revascularization in patients with ischemic cardiomyopathy. Equally important, FDG imaging detects inflammation-associated cardiac device infection and infiltrative processes, including cardiac sarcoidosis. Second, linking the observations obtained from preclinical and human studies offers an opportunity for serial in vivo monitoring of myocardial metabolism in preclinical models of disease. For example, the demonstration that metabolic perturbations appear to be necessary for development of hypertension-induced left ventricular hypertrophy and dysfunction in preclinical models (Figure 4) provides corroborative information on metabolic changes as determinants of left ventricular remodeling in humans. Conversely, observations obtained in humans can also provide important information. For example, the demonstration of sexual dimorphism in the myocardial metabolic response to obesity and type 2 diabetes mellitus in humans provides an impetus to perform preclinical studies with stratification into male and female subgroups.[51,52] Lastly, the integration of the myocardial metabolic patterns associated with the human disease with those observed in relevant preclinical models should facilitate discovery and development of new therapeutic approaches for metabolic modulation. Please refer to the appropriate section in the Appendix.

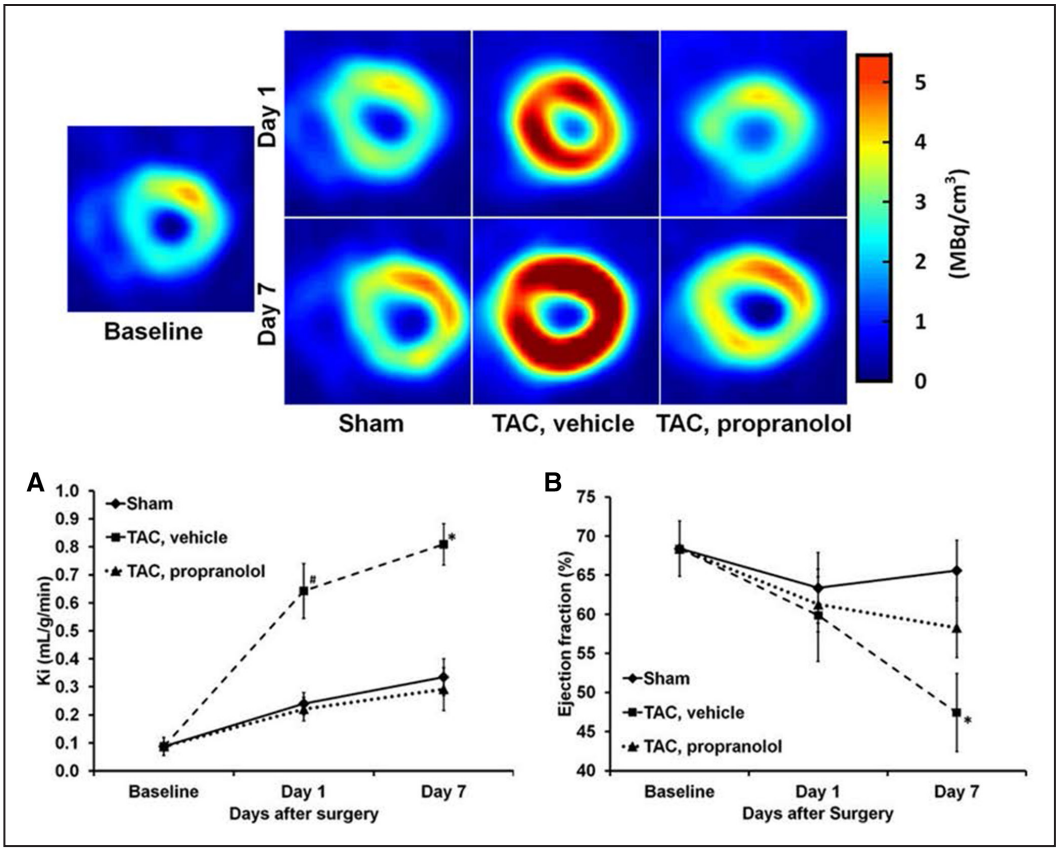

Figure 4. Top, End-diastolic transverse positron emission tomography images for sham, transverse aortic constriction (TAC), and TAC mice treated with propranolol at baseline and on day 1 and day 7 after surgery. The images show an increase in uptake of the glucose tracer analog [^{18}F]2-deoxy-D-glucose (FDG) in TAC mice starting at day 1, indicative of the metabolic adaptation in pressure-overload left ventricular hypertrophy. **Bottom**, **A**, Measured rates of myocardial FDG uptake in vivo, showing K_i (in mL·g^{-1}·min^{-1}). **Bottom, B,** Measured left ventricular ejection fraction from dynamic gated positron emission tomography images in vivo. All values are mean ± SEM. *P<0.05 vs baseline, TAC-propranolol, and sham groups. Reprinted from Zhong et al[79] with permission. Copyright © 2013, the Society of Nuclear Medicine and Molecular Imaging, Inc.

^{31}P and ^{1}H MRS (NMR)

General

Both ^{31}P and ^{1}H spectroscopy offer an opportunity to assess serially metabolic activity in the intact heart and can be used both ex vivo and in vivo. Although MR imaging predominantly relies on ^{1}H nuclei (protons) in water (H_2O) molecules as its signal source, cardiac MRS (also referred to as NMR or NMR spectroscopy) enables the study of many other nuclei and is currently the only available method for the noninvasive assessment of cardiac metabolism without external radioactive tracers (see references 80–82 for review). Atomic nuclei of greatest interest are ^{31}P, ^{13}C, and ^{1}H (ie, ^{1}H metabolites other than water).

Although in principle, many clinical questions can be answered with cardiac MRS, the method has not yet fulfilled its potential in clinical practice because of fundamental physical limitations: nonproton nuclei have a much lower MR sensitivity than ^{1}H, and metabolites interrogated with MRS are present in concentrations several orders of magnitude lower (millimolar range) than those of ^{1}H nuclei in water (110 mol/L). Therefore, cardiac MRS signals are typically 10^3 to 10^6 times weaker than those in conventional MR imaging, which leads to poor spatial (≈30ml) and temporal (>15 minutes) resolution and high measurement variability (≥15%). Implementation of MRS in clinical practice will only become a reality if MRS signals can be augmented substantially.

The ^{31}P MRS Technique

Experimental Applications

The upper left panel of Figure 5 shows a ^{31}P-MR spectrum from an isolated beating rat heart. A cardiac ^{31}P spectrum shows 6 resonances, the 3 ^{31}P-atoms of ATP (the right shoulder of the alpha-P-ATP peak represents NAD$^+$), phosphocreatine, inorganic phosphate, and monophosphate esters (mostly AMP and glycolytic intermediates); that is, ^{31}P metabolites present above a certain concentration threshold of ≈0.6 mmol/L and free in solution. In combination with chemical or ^{1}H MRS determination of total creatine, the free cytosolic ADP concentration and the free energy change of ATP hydrolysis (ΔG [kJ/mol]) can be calculated. Because of "chemical shift" (explained by slight differences in the electrochemical environment of the nuclear spins), different metabolites resonate at distinct frequencies, which enables their discrimination from each other. Intracellular pH is determined from the chemical shift between inorganic phosphate and phosphocreatine, which is pH sensitive.[84] The area under each resonance is directly proportional to the number of each ^{31}P-nucleus species in the heart, and

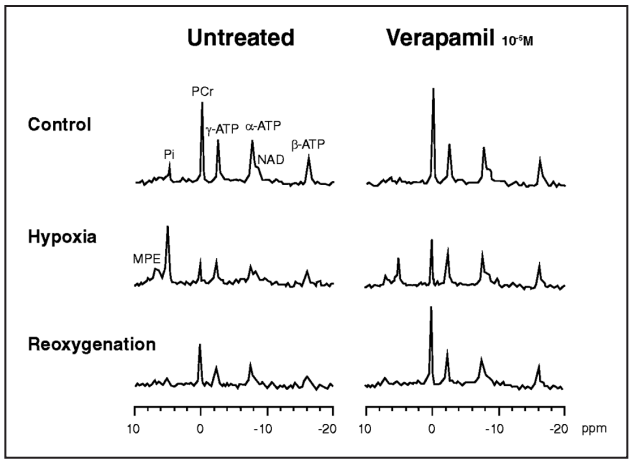

Figure 5. Serial ^{31}P magnetic resonance spectra from a heart treated with verapamil during control and an untreated perfused rat heart, as well as at the end of 30 minutes of hypoxia and at the end of 30 minutes of reperfusion. During hypoxia, phosphocreatine (PCr) and ATP decrease, whereas inorganic phosphate (Pi) increases and an additional resonance (monophosphate esters [MPE], mostly glycolytic intermediates and AMP) appears. During reoxygenation, PCr and Pi recover, but ATP does not. Verapamil pretreatment attenuates these changes. NAD indicates nicotine adenine dinucleotide. Reprinted from Neubauer et al[83] with permission from Elsevier. Copyright © 1989, Elsevier Ltd.

metabolite resonances are quantified by measuring peak areas. Relative metabolite levels, such as the phosphocreatine to ATP ratio, are calculated directly, whereas determination of absolute metabolite concentrations requires comparison to an external reference standard with a known amount of ^{31}P. The most important advantage of MRS over traditional biochemical assays is that MRS measurements are noninvasive, spectra can be acquired sequentially, and the metabolic response to ischemia, hypoxia, or inotropic modulation can be followed over time. Thus, each heart serves as its own control (spectra displayed in Figure 5). With ^{31}P MRS, the energy-rich phosphate state of the heart can be monitored sequentially, at baseline, during workload changes, during ischemia/reperfusion, or in models of chronic hypertrophy and heart failure. Furthermore, with the magnetization (saturation) transfer method,[85] the rate and velocity of the creatine kinase reaction, a measure of ATP transfer from mitochondria to myofibrils, can be assessed.

^{31}P MRS is also performed in the heart in vivo, in rodents, in large animals, and in humans (Figure A2 in the Appendix). In an open-chest model, a surface coil is fixated over the heart. In the closed-chest model, additional localization techniques are needed ("^{13}C MRS (NMR)"). In large animal models, the transmural variation of high-energy phosphate levels can be assessed, with changes during stress generally greater in the subendocardial than the subepicardial layer.[86] Of note, the effects of general anesthesia on high-energy phosphate metabolism have never been assessed. The clinical applications of ^{31}P MRS are further discussed in the Appendix.

The ^{1}H MRS Technique

^{1}H MRS is another technique to assess cardiac metabolism. Protons possess high natural abundance and the highest MR sensitivity of all MR-detectable nuclei. Most, if not all, metabolites have ^{1}H nuclei, and those typically detected by ^{1}H MRS include creatine, lactate, carnitine, taurine, and -CH_3 and -CH_2-resonances of lipids.[82,87] By means of the oxymyoglobin and deoxymyoglobin resonances, tissue deoxygenation can also be measured. Isolated heart studies have assessed many of these resonances, and total creatine can be measured in hearts of large animals. Currently, no information on the effects of different buffers on ^{1}H MRS spectra from the heart is available.

Human ^{1}H MRS is typically performed by single-voxel techniques such as stimulated echo acquisition mode (STEAM)[88] or point-resolved spectroscopy (PRESS),[89] which are synchronized with the heartbeat. In vivo ^{1}H MRS also necessitates the compensation of respiratory motion either through navigator techniques or by scan acquisition during breath hold. Metabolite signals from a 2- to 15-mL volume can be obtained in <10 minutes. A human cardiac ^{1}H MRS spectrum acquired in a single breath hold (Figure A3 in the Appendix) shows the resonances for (mostly but not completely suppressed) water, TG (fat; lipid), and total creatine. Most human cardiac ^{1}H MRS studies have assessed changes in these metabolites.[87,90] The new technique of chemical exchange saturation transfer relies on the signal transfer between water and amine protons of creatine, achieving an assessment of free creatine content in the heart with a signal to noise ratio ≈70-fold greater than that of conventional ^{1}H MRS, which offers the prospect of noninvasive high-resolution measurement of cardiac metabolism in humans.[91]

In addition to the limitations to signal to noise ratio discussed above, technical challenges for ^{1}H MRS include the need for suppression of the strong ^{1}H signal from water; the complexity of ^{1}H spectra with overlapping resonances, many of which remain to be characterized; and the contamination of intramyocardial lipid signals by epicardial fat. To avoid this, human ^{1}H MRS studies typically interrogate the interventricular septum. Despite this limitation, ^{1}H MRS of intramyocardial TG is used to assess cardiac steatosis and the development of impaired glucose tolerance in humans and is linked to early indices of impaired contractility when combined with tagged cardiac MR imaging in animals.[92,93] A major effort, including advances in coil and sequence design, higher field strength, and standardization of protocols, is still required to bring cardiac MRS into clinical practice.

^{13}C MRS (NMR)

Carbon-13 NMR (^{13}C NMR) spectroscopy provides comprehensive insights into mechanisms for the metabolic basis of cardiac dysfunction.[94] The low natural abundance of the stable isotope ^{13}C can be used, conceptually, like a radiotracer, albeit not at tracer levels but rather at physiological concentrations. Every energy-containing substrate can be enriched with ^{13}C at specific sites and administered without concern about radiation containment. Detection of ^{13}C by MR identifies specific metabolites, as well as specific carbon sites within the metabolite molecules. The poor sensitivity for detecting ^{13}C by MR can be overcome by the methods discussed below.

Physical Principles of ^{13}C Enrichment and NMR Detection

The ^{13}C NMR spectrum displays signal as a function of chemical shift. The chemical shift provides highly specific information about the site of ^{13}C labeling in a molecule and the structure of the parent molecule. An advantage of ^{13}C is the very wide chemical shift dispersion, ≈200 ppm compared with ≈10 ppm for ^{1}H. Consequently, molecules that are structurally very similar and difficult to resolve by ^{1}H NMR are almost always easily separated in the ^{13}C NMR spectrum. Another difference of ^{13}C detection compared with ^{1}H is overall sensitivity. NMR (or MRS), of all atomic nuclei, is generally insensitive because nuclear polarization is poor. For practical purposes, the inherent sensitivity of nuclei is typically referenced to ^{1}H with a value of 1. The magnetogyric ratio, or γ (roughly the "strength" of the ^{13}C nucleus), is approximately one-fourth that of ^{1}H. Because sensitivity scales approximately as γ^3, ^{13}C has approximately 1/64th the sensitivity of ^{1}H. This fact, combined with the low natural abundance of ^{13}C (0.011), means that the overall sensitivity for detecting ^{13}C is $\approx 0.011 \times 1/64 = 0.00017$ relative to ^{1}H. The application of higher magnetic fields enables greater signal detection. For hearts in vivo, isolated perfused hearts ex vivo, and tissue extracts in vitro, the field strengths of NMR magnets for ^{13}C analysis of the heart range from clinical fields of 3.0 T all the way to 18.8 T.

The detection of adjacent ^{13}C nuclei via spin-spin coupling provides metabolic information beyond the simple chemical shift data. Spin-spin coupling between covalently bonded nuclei, with net spin, produces splitting of the NMR signal from an observed nucleus into separate peaks or multiplets of that resonance signal because of the influence of distinct energy states of covalently bonded nuclei on the observed nucleus. These 2 features of ^{13}C NMR, chemical shift and spin-spin coupling, provide direct information about the metabolism of a compound that is not accessible by radiotracers (Appendix).

Although spin-spin coupling between ^{13}C nuclei is advantageous for detailing patterns of substrate oxidation in the heart, ^{13}C-^{1}H coupling produces overlapping multiplets within the resonance signals from the ^{13}C nuclei that complicate ^{13}C NMR spectra and can confound analysis. The complexity is remedied by applying a second radiofrequency at the resonance frequency of ^{1}H to maintain a saturated spin state in a process called decoupling. Decoupling collapses the multiplet structures of the NMR peaks from the ^{1}H-coupled ^{13}C nuclei, which simplifies interpretation of the ^{13}C NMR data set. Although technically easy to apply, attention to sample heating is required because of the radiofrequency energy deposition.

Hyperpolarized ^{13}C NMR

An emerging technique that circumvents the low sensitivity and the need for decoupling with a conventional NMR approach is hyperpolarized ^{13}C NMR. Hyperpolarization refers to any one of a number of procedures[95] to temporarily redistribute the polarization state of nuclei, a concept initially demonstrated in noble gases.[96,97] One method, dynamic nuclear polarization, involves freezing a sample of ^{13}C-enriched material at ≈1.4°K in a glass containing a stable radical. In a static magnetic field of 3 to 5 T, electrons in the sample are polarized >90%. Exposure to microwaves forces transfer of this electron polarization to the ^{13}C nuclei, which enables increases in ^{13}C polarization >10 000-fold.[97–99] The hyperpolarized state is only temporary, so sample transfer and infusion must be performed quickly, along with rapid imaging sequences, within 2 to 3 minutes. Use of hyperpolarized ^{13}C NMR holds experimental constraints, but the large increases in signal to noise ratio that are achieved with dynamic nuclear polarization have enabled ^{13}C NMR to be performed directly on the heart in vivo.[100–103] (The Appendix contains further detail.)

Conventional ^{13}C NMR Evaluation of Cardiac Metabolism

^{13}C NMR Measurements of Cardiac Substrate Selection Ex Vivo and In Vivo

A simple approach to evaluate cardiac oxidative metabolism involves a single ^{13}C NMR spectrum to assess entry of various ^{13}C-enriched fuels into the Krebs cycle[104–109] (Figure 6). Direct detection of acetyl-CoA would be desirable, but concentrations of acetyl-CoA (and most of the Krebs cycle intermediates) are too low and not detectable by NMR methods. However, glutamate is present in high concentration in heart tissue and is in chemical exchange with intermediates of the Krebs cycle. Therefore, although the interconversion between Krebs cycle intermediates and glutamate is not instantaneous, ^{13}C NMR detection of the ^{13}C enrichment of glutamate provides a read out of intermediate isotopomers and thus the isotopic enrichment of acetyl-CoA entering the Krebs cycle (Figure 6). As a consequence of spin-spin coupling (described in Physical Principles of ^{13}C Enrichment and NMR Detection), ^{13}C NMR detects patterns of ^{13}C labeling in these specific molecules that reflect the distribution of the isotope at multiple carbon positions within otherwise identical molecules, called *isotopomers* (Appendix).

If the oxidation of only a single substrate or set of substrates as a single group is of interest, this analysis can be further simplified by using substrates that exclusively enrich acetyl-CoA at the 2-carbon site alone.[107,108,111–113] Such labeling patterns for physiological substrates for the heart include [1,6-$^{13}C_2$] glucose (5–10 mmol/L), [3-^{13}C] lactate (0.5–2 mmol/L), [3-^{13}C] pyruvate (0.1 mmol/L), and [2,4,6,8,10,12,14,16-C_8] palmitate (0.2–1.2 mmol/L) and [2,4,6,8,10,12,14,16,18-C_9] oleate (0.2–1.2 mmol/L). More detailed analyses can also be applied to assess the relative activities of anaplerotic pathways.[114–117]

NMR-based detection of glutamate isotopomers also provides assessment of the nonenriched fraction of acetyl-CoA entering the Krebs cycle. The amino acid alanine is in isotopic equilibrium with the much lower-level intracellular pyruvate, and the methyl group of alanine can be detected by in vitro ^{1}H NMR as a separate signal emanating from the ^{13}C-enriched methyl groups produced from exogenous ^{13}C glucose or unenriched, endogenous ^{12}C glucose from glycogen.[111,118] Experimentation with ^{13}C LCFAs also exposes the fraction of nonenriched acetyl-CoA that originates from the nonenriched

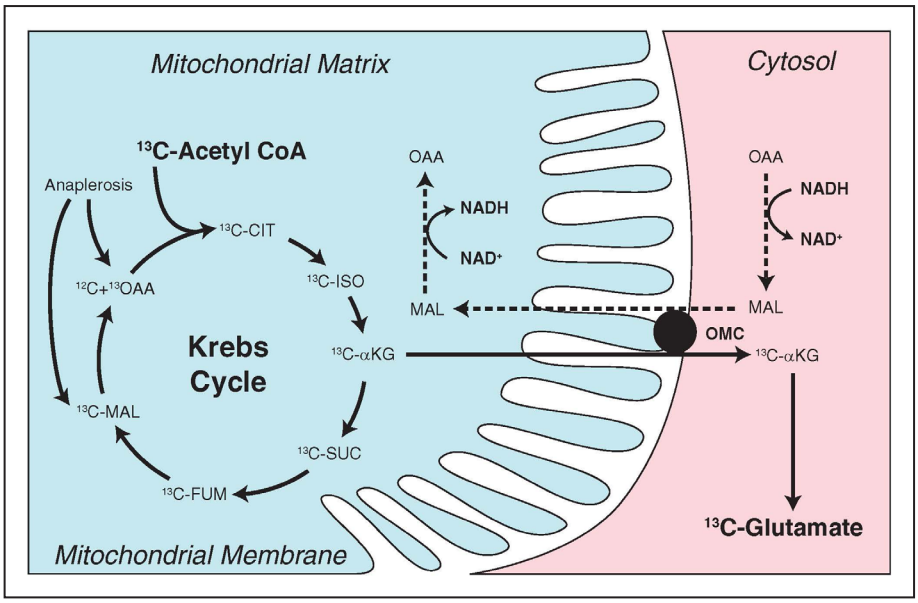

Figure 6. Abbreviated schematic of carbon-13 (^{13}C) isotope labeling for nuclear magnetic resonance detection of enriched glutamate. Catabolism of ^{13}C-enriched metabolic precursors contributes to [^{13}C]acetyl-CoA, which enters the Krebs cycle via the citrate synthase reaction. Oxidation of ^{13}C citrate forms ^{13}C-enriched α-ketoglutarate (αKG). Either ^{13}C αKG is then oxidized to form ^{13}C succinate, which introduces the ^{13}C into the second span of the Krebs cycle, or ^{13}C αKG is exported across the mitochondrial membrane to the cytosol via a rate-determining process through the reversible αKG (or oxoglutarate) malate (MAL) carrier (OMC). Depending on the experimental protocol, various carbon positions with the metabolic intermediates can be isotopically labeled from the delivery of differential ^{13}C enrichment schemes within an individual precursor or a mixed supply of fuels for acetyl-CoA production and oxidative metabolism (fats, carbohydrates, and ketones). The carrier-mediated exchange of αKG for MAL has been shown to be detectable and to introduce a separable rate to the formation of ^{13}C glutamate that is coupled to but distinct from flux through the Krebs cycle. This exchange can occur in tandem with exchange through the unidirectional, glutamate-aspartate carrier and associated transamination reactions (not shown) to form the MAL-aspartate shuttle, the mechanism for transferring reducing equivalents from the cytosol to the mitochondria by coupling to NAD^+/NADH redox reactions. CIT indicates citrate; FUM, fumarate; ISO, isocitrate; NAD, nicotinamide adenine dinucleotide; OAA, oxaloacetate; and SUC, succinate. Reprinted from Lewandowski et al[110] with permission of Springer. Copyright © 2013, Springer Science + Business Media, LLC.

endogenous FAs stored in the cell as TG. If the approach is extended to include LCMS analysis of fractional enrichment of the stored TG pool in the cell, which also becomes ^{13}C enriched by the esterification of any exogenous ^{13}C LCFAs that are delivered, then a total accounting of all sources, both exogenously delivered and endogenous stored pools, can be implemented successfully.[111] As discussed previously, under such circumstances, the measured oxidation of labeled fats can actually decrease, despite increased rates of β-oxidation.

Approaches for NMR detection of ^{13}C enrichment of glutamate and glutamate isotopomers can be extended to analysis of tissue extracts of frozen myocardial samples isolated from animals that are supplied either single or multiple ^{13}C-enriched substrates in vivo.[112,113,117,119–124] Coronary artery infusion of saline solution containing ^{13}C-enriched fats or carbohydrates allows precise measurements of cardiac substrate utilization in the open-chest animal model, and more recently in the conscious, closed-chest pig model that is fully instrumented.[113,120] However, by definition, metabolic flux rates require multiple time points and therefore multiple sets of NMR spectra.

Quantifying Metabolic Flux Rates in Isolated Hearts With Conventional ^{13}C NMR Detection

Dynamic data sets from sequential ^{13}C NMR spectra are well suited for monitoring Krebs cycle flux, rates of LCFA entry into oxidative metabolism, and rates of LCFA esterification into the stored TG pool.[111] Sequential ^{13}C NMR spectra from hearts supplied physiological concentration of ^{13}C-enriched substrates over the time course of progressive glutamate enrichment provide a quantitative measure of Krebs cycle flux in the intact beating heart in both normal and pathological states. Such experiments have been implemented in the in vivo heart of open-chest animal models using surface coils for NMR detection, but the bulk of work using this approach has employed the isolated perfused heart model.[116,119,125–137]

The premise of this experimental scheme to provide quantitative information on Krebs cycle and substrate oxidation rates lies in the NMR detection of the relative rates of ^{13}C incorporation at the primary 4-carbon position of intermediates within the first span of the cycle, as defined by Randle et al,[138] and at the secondary 2- and 3-carbon sites of intermediates in the second span (in equal probability), as reflected in the progressive ^{13}C enrichment of the NMR-detectable glutamate (Figure 6). Precise details of the processes of progressive enrichment of glutamate with ^{13}C in relation to Krebs cycle activity are available in the literature.[107–109,125,130–132,139–141]

The production of ^{13}C-enriched glutamate via ^{13}C substrate oxidation through the Krebs cycle is influenced by cotransport of α-ketoglutarate (2-oxoglutarate) and malate through the oxoglutarate-malate carrier on the mitochondrial membrane and has been associated with the level of oxoglutarate-malate carrier expression in the heart.[126] Therefore, depending on the pathophysiological provenance of the heart, the α-ketoglutarate to glutamate interconversion imposes a

rate-determining step to the assessment of oxidative flux in the heart that relies on glutamate enrichment rates. Kinetic analyses can account for these processes and provide additional insights into metabolic regulation (Appendix).[125,126,131,137]

To sum up, ^{13}C NMR is widely used to study cardiac metabolism in isolated rodent hearts, largely for monitoring the relative rates of oxidation of various substrates in the Krebs cycle when multiple substrates are available and for kinetic studies of flux rates in the intact beating heart (Appendix). Both static measurements of relative substrate oxidation by the heart and measurements of metabolic flux rates from serial detection of the rates of ^{13}C incorporation into key metabolites have made ^{13}C NMR a tool to elucidate otherwise elusive processes that occur within the myocardium of the intact functioning heart. Unique among methods that use carbon isotopes, well-designed NMR experiments can provide quantitative information about both metabolic rates and transport rates across the sarcolemma and mitochondrial membranes. In the future, a possible approach to personalized care of heart disease may be based on assessment of cardiac fuel metabolism by ^{13}C NMR.

Stable Isotope Labeling by GCMS/LCMS

Mass spectrometry (MS) coupled to gas chromatography or liquid chromatography is also used to investigate heart metabolism with stable isotopes. It measures flux rates through metabolic pathways, such as glycolysis, glucose and FA oxidation, the Krebs cycle, and anaplerosis (for reviews, see references 142–145).

Use, Utility, and Advantages of Stable Isotope Labeling by GCMS/LCMS

The past 25 years have witnessed the development of isotopomer analysis, including mass isotopomer analysis based on MS, and of positional isotopomer analysis based on MRS. These techniques have been used extensively in perfused rat and mouse hearts to trace the contribution of ^{13}C-labeled fuels to ATP production and to anaplerosis (replenishment of Krebs cycle intermediates). There is generally good agreement between fluxes calculated by the 2 techniques.[143] The 2 techniques provide distinct yet complementary information. MRS allows identification of the position of labeled atoms in molecules but detects only those atoms that are labeled (^{13}C, ^{15}N). MS is more sensitive than MRS and enables assessment of the labeling of whole molecules or fragments of molecules. Both techniques have been used to trace processes and fluxes in intermediary metabolism of the heart, with emphasis on the contributions of fuels to energy substrate metabolism and anaplerosis. Fluxes are assessed by use of (1) the labeling of acetyl-CoA or the acetyl moiety of citrate, assayed by MS (142–145) versus (2) the labeling of C-4,5 of glutamate, a proxy of acetyl-CoA labeling, assayed by MRS.[146] In addition, MS assesses other complex processes in the intact heart, such as (1) simultaneous lactate and pyruvate release (from glycolysis), lactate/pyruvate uptake (for oxidation in the Krebs cycle), and pyruvate partitioning between oxidative decarboxylation and carboxylation; (2) the reversibility of the isocitrate dehydrogenase reaction (recently renamed as reductive carboxylation of glutamate); (3) fumarate metabolism though the reductive pathway; (4) the partitioning of LCFAs between mitochondrial, peroxisomal β-oxidation, and TG synthesis (for reviews, see references 142, 144, and 145); (5) the contribution of glutamine to anaplerosis[147]; and (6) the source of acetyl-CoA oxidized in the Krebs cycle[148,149] or used for FA elongation.[150] These labeling studies using MS have been conducted in isolated rat and mouse heart study models, as well as in pig hearts,[142–145] but they may also be applied to isolated cardiomyocytes (although rates of energy substrate metabolism are lower than in the intact beating heart).

Caveats

Most stable isotopic investigations based on MS or MRS are subject to limitations and artifacts. On the one hand, the fractional contribution of energy substrates to energy metabolism when using equations based on ^{13}C-labeled isotopomer balance assumes achievement of a metabolic steady state, which requires stable cardiac contractile function, as well as an isotopic steady state.[143,144] On the other hand, analysis of substrate oxidation by ^{13}C MRS in the heart is well suited to non–steady state conditions such as ischemia or stunning, in which metabolic and isotopic steady states cannot be assured.[141,151] However, monitoring of the labeling or delabeling of metabolite pools can provide useful kinetic information on the process under study or lead to the identification of a new pathway.[144]

A frequent problem encountered in any isotopic study is the generation of secondarily labeled substrates that may alter the labeling pattern of the metabolites under investigation. An example is the formation of labeled lactate (from glycolysis) in the ex vivo heart model perfused with recirculating buffer containing labeled glucose. Another frequent problem is that the labeling of a product can result from a net metabolic flux, isotopic exchange processes, or both (the combination typically being the most common). For instance, labeling of a metabolite through isotopic exchange, without a net metabolic flux, can occur for ketone bodies in heart or muscle, a process referred to as pseudoketogenesis, because of the reversibility of the thiolase and 3-oxoacid transferase reactions.[152] For a mathematical biologist, labeling or delabeling via isotopic exchanges provides information on the metabolic network. However, for a metabolic biologist, isotopic exchanges do not reflect a metabolic load (unless they occur via ATP-utilizing mechanisms such as substrate cycling). In 1969, Weidemann and Krebs had already cautioned that "the fates of the label does not allow predictions to be made on the net fate of the labeled metabolites."[153] This caveat is often forgotten.

When measuring isotopic enrichment using MS, one must also make sure that the instrument is tuned so that the measured mass isotopomer distributions (MIDs) of a standard of pure analyte and unlabeled analyte in a tissue extract are very close to the theoretical distribution. How to manage substantial deviations of a measured natural MID from its theoretical profile is still an evolving field.[154–158] Most investigators use calculation routines to deduct the MID of unlabeled acetyl-CoA from the measured MID of samples of multilabeled acetyl-CoA. Some investigators prefer to include the atomic compositions of the analytes and the natural enrichment of each atom species into equations constructed to convert the measured MIDs of labeled samples to metabolic data.[159]

To sum up, the use of stable isotope labeling by GCMS/LCMS for investigations of cardiac metabolism provides some

unique advantages over other labeling approaches because of its sensitivity. This approach assesses substrate flux through complex metabolic processes, beyond substrate fuel selection for energy provision, the role and regulation of which remain to be better understood. These processes may consume energy through substrate cycling, affect redox balance or proton production, or result in the formation of cytotoxic metabolic end products; thus, they are critical to the overall energy balance, efficiency of energy transfer, and metabolic integrity of the heart.

Turnover of Intracellular Macromolecules: Proteins, Glycogen, and TGs

The turnover of intracellular macromolecules (proteins, glycogen, and TGs) is an integral part of cardiac metabolism, because all structural and functional components of the cardiomyocyte are in a state of flux. In other words, steady state concentrations of macromolecules do not reveal their respective rates of synthesis and degradation. This idea is not new but is rarely considered. It dates back to the concept of the "dynamic state of body constituents" revealed by stable isotopes. Brief historical notes on the use of stable isotopes can be found in the Appendix.

Protein Turnover

Maintenance of intracellular protein homeostasis comprises the targeted degradation of damaged (or unneeded) proteins via either the ubiquitin proteasome system,[160] the autophagy lysosome system,[161,162] or both,[163] and it includes the resynthesis of new and functional proteins.[164] This section describes approaches to assess protein turnover in the whole heart.

Quantification of myocardial protein turnover is a first step for assessing remodeling of the heart in response to hemodynamic, neurohumoral, and metabolic stress (including oxidative stress and oxygen deprivation). Proteotoxicity is the result of dysregulated protein turnover and is increasingly implicated in contractile dysfunction and heart failure.[165,166]

Several tracer techniques are available to assess changes in myocardial protein synthesis and degradation in the intact heart, both in vivo and ex vivo. The methods are based on compartmental analysis and require the administration of labeled compounds and their biosynthetic incorporation into newly synthesized heart muscle proteins.[164,167] Figure 7 depicts the principle. In pulse-chase experiments, which remain the gold standard for assessment of myocardial protein turnover, it is a prerequisite that the tracer amino acid itself is not subject to synthesis and degradation, a condition met by the aromatic amino acids phenylalanine and tyrosine.[168] Further discussion of the dynamics of myocardial protein turnover can be found in the Appendix.

Contemporary methods to assess myocardial protein turnover use some of the same stable isotopes already used in the late 1930s (Appendix). Synthesis to degradation ratio MS measures the relative dynamic turnover of specific proteins in cellular systems.[169] In this approach, stable isotope labeling is used to calculate a synthesis to degradation ratio that reflects the relative rate at which ^{13}C is incorporated into individual proteins in the cell. Recent approaches for the assessment of cardiac proteome dynamics have deployed deuterated H_2O (^2H_2O, or "heavy water")[170,171] in combination with high-resolution MS and bioinformatics tools. The approach is applicable to a wide spectrum of proteins and permits estimation of $t_{1/2}$ for individual proteins before and after a pharmacological intervention. The stable isotope methods offer a quantitative and longitudinal assessment of the plasticity of the heart and have been translated to human studies,[171] although they still await independent validation. Recent work has also shown the utility of ^2H_2O for assessment of mitochondrial proteome dynamics.[172] The field is likely to grow.

Glycogen Turnover

The fetal heart and the hibernating myocardium are rich in glycogen[173–175]; in the adult heart, glycogen serves as a critical endogenous source of energy for contraction. For example, in response to increases in workload (eg, physical activity associated with the fight-or-flight response), rapid activation of glycogenolysis provides an instantaneous source of glycosyl units (in the form of glucose 6-phosphate) for ATP generation (via glycolysis, Krebs cycle, and oxidative phosphorylation) to meet energetic demands.[176–178] Conversely, during myocardial infarction, the ischemic region of the myocardium relies solely on glycogen as an anaerobic fuel for maintenance of essential cellular function.[179–181] Consistent with the need to use and replenish glycogen stores, myocardial glycogen content oscillates as a function of time of day, in part because of

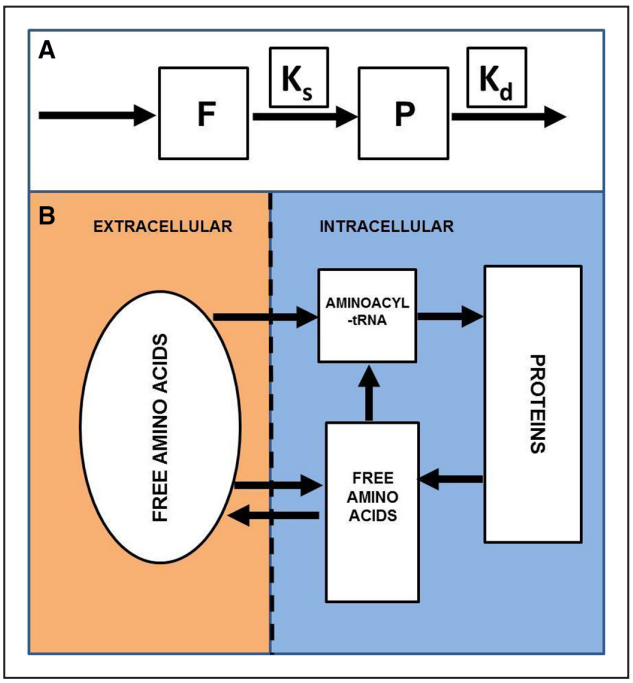

Figure 7. Compartmental model of myocardial protein turnover. **A**, The theoretical model for the precursor/product relationship described in the equation under Measuring Metabolism. F and P refer to free amino acids in the precursor and bound amino acids in the protein compartments, respectively. K_s and K_d are first-order rate constants for the rates of transfer of amino acids in and out of the protein compartment. **B**, The same compartments, including known pathways of precursor and product uptake and release from myocardial proteins. tRNA indicates transfer ribonucleic acid. Modified with permission of FASEB from Samarel[164]; permission conveyed through Copyright Clearance Center, Inc. Copyright © 1991, FASEB.

changes in neurohumoral factors associated with sleep/wake and fasting/feeding cycles.[58]

Glycogen turnover has been assessed in the heart through use of both radiolabeled (^{14}C and ^{3}H) and stable (^{13}C) isotope tracer pulse-chase studies in the rat. After initial incorporation of ^{14}C-labeled glycosyl units in the glycogen pool (through perfusion in the presence of ^{14}C-glucose for 40 to 45 minutes; pulse), ex vivo working rat hearts are perfused with ^{3}H-labeled glucose (chase), for simultaneous assessment of glycogen synthesis (incorporation of ^{3}H glucose into glycogen) and breakdown (^{14}C release in the form of labeled lactate and CO_2) during the chase period.[178,182] This technique has revealed that (1) under aerobic conditions, myocardial glycogen is preferentially oxidized; (2) during an acute increase in energetic demand, myocardial glycogen is used initially to a greater extent than exogenous glucose; (3) after glucose uptake, there is not an obligate requirement for glycosyl units to enter the glycogen pool before entry into glycolysis; (4) the last-on, first-off rule for glycosyl units within the glycogen particle does not necessarily apply for the heart; and (5) insulin potently attenuates rates of myocardial glycogen turnover.[176–178,182] The latter has also been observed in vivo for the rat heart by use of a complementary ^{13}C NMR protocol, wherein ^{13}C-labeled glycosyl units that were incorporated into glycogen during the pulse period remained within glycogen throughout the chase period (50 minutes) when insulin was present.[183]

Although of uncertain physiological significance, rat heart perfusions with [U-$^{13}C_3$]glycerol have revealed de novo glycogen synthesis in the heart, which underscores the power of ^{13}C NMR techniques.[184] Myocardial glycogen turnover can also be estimated though simultaneous assessment of net ^{14}C-labeled glucose into glycogen and total glycogen content; this modified protocol has suggested that specific perfusion conditions (such as simultaneously elevated insulin and acetoacetate levels) may accelerate glycogen turnover in the rat heart,[69] and exposed the dynamic nature of myocardial glycogen turnover. Lastly, elevated levels of GLUT4 during reperfusion facilitate the replenishment of glycogen pools after myocardial ischemia, which further underscores the need for methods to assess glycogen metabolism in the heart.[185]

TG Turnover

Interest has grown regarding the active biochemical and physiological roles of TG in the cardiomyocyte as a source of lipid-based signaling species (eg, nuclear receptor activation for the induction of metabolic target genes). TGs also serve as a major source of FAs undergoing β-oxidation from turnover of the TG pool, and in contrast to prior notions of a static pool of stored lipid, rates of TG turnover are substantial compared with oxidative rates in the heart and impact the levels of acyl derivatives that are physiologically active and potentially lipotoxic.[111,186–192]

Radiolabeled (^{14}C) LCFAs are used to examine turnover within the steady state triglyceride pool. Although yielding biologically significant results, this method presents difficulty with regard to quantifying and accounting for the contribution of unlabeled metabolites to such rate measurements.[176,193–196] Radioisotope labeling of TG has been applied to either a single end point with the assumption of a linear labeling rate or at multiple time points that require sets of separate hearts that are collected at each time point. In the instance of any deviation from an apparent linear rate of isotopic labeling, which is actually the initial rise of an exponential, the lack of multiple time points obtained from a single heart over time can be limiting.[187,197] Nonetheless, the application of a classic pulse-chase protocol with radioisotope-labeled LCFAs has exposed the cardiac response to different pathological conditions. For example, hearts can be perfused with ^{14}C-LCFA for baseline triglyceride labeling and then, after a period of ischemia, the same hearts can be reperfused with tritiated (^{3}H) LCFA to examine TG labeling in the postischemic heart.[195,196]

Alternatively, ^{13}C NMR is a valuable tool in examining TG turnover rates under steady state conditions within the intact beating heart perfused with ^{13}C LCFA.[111,187,188,190,197] The ability to monitor the ^{13}C enrichment rates of both glutamate and TG within the same heart by ^{13}C NMR spectroscopy enables simultaneous assessment of the reciprocal processes of FA oxidation and esterification in the same experiment. In many instances, the quantitative study of intracellular lipid dynamics using stable isotopes in intact hearts requires conventional proton-decoupled ^{13}C NMR spectroscopy to define the subcellular events of FA transport into the cardiomyocyte and turnover within the neutral lipid pool, although TG turnover can also be measured in mouse heart by dynamic ^{13}C edit-^{1}H observe NMR with determination of FA oxidation by ^{13}C isotopomer analysis.[198] In addition, the advantages of the chemical specificity and kinetic analysis of the ^{13}C enrichment rates detected by NMR, with end-point, LC/MS quantification of TG ^{13}C enrichment and content of low, non–NMR-detectable acyl intermediates, provide flux rate data inherent to but hidden within the noninvasive ^{11}C PET approach.[199–202]

Measurements of TG turnover with ^{13}C NMR spectroscopy of the heart are possible with the introduction of ^{13}C-enriched LCFAs into the buffer supplied to the isolated perfused heart. ^{13}C NMR signal is obtained from the progressive enrichment of intramyocellular TG caused by esterification of ^{13}C LCFA into TG, as detected at the resonance frequency assigned to the methylene carbons that correspond to 31 ppm within the NMR ^{13}C spectra.[203] The detection is specific to TG, because the concentration and abundance of the other acyl intermediates that may become ^{13}C enriched within the cell are too low to contribute NMR signal. Because ^{13}C is 1.1% abundant in nature, an appreciable background ^{13}C NMR signal arises because of the many carbon nuclei within the heart. Therefore, as in all ^{13}C NMR detection experiments performed on the intact organ, a natural abundance of ^{13}C NMR spectrum is collected for subsequent digital subtraction from spectra collected during the isotopic enrichment protocol. In this manner, sequential ^{13}C NMR spectra are then collected from the intact heart to determine the rates of carbon isotope incorporation into the TG pool. After the detection of the progressive isotopic enrichment of TG, the heart is freeze-clamped for end-point quantification of TG and determination of final enrichment levels with MS. To provide TG turnover rates, the experiments must be performed under steady state conditions in which the content of the total TG pool does not change over the course of the detection period.

Directly Assessing Cardiac Metabolism In Vivo

Measurements of energy substrate metabolism by withdrawal of paired blood samples from aorta and coronary sinus in vivo are well established[204] and offer some advantages over other methods. First, body homeostasis is preserved while the heart remains intact and physiologically perfused. Second, confounding effects of artificial experimental conditions are minimal. However, in contrast to isolated heart preparations, there is only limited control over the concentration of circulating cardiac metabolic substrates and hormones, and the heart remains under the control of the autonomic nervous system. All of these factors influence cardiac work, coronary flow, and substrate selection. Therefore, they must be closely monitored and possibly stabilized. It is also necessary to keep the catheter, in the coronary sinus, in a stable position during the entire procedure. Lastly, the rate of substrate uptake and oxidation can only be determined reliably with accurate real-time measurements of total coronary blood flow. The popular thermodilution-based measurement of coronary sinus flow is not always accurate, whereas real-time measurements of coronary arterial flow are limited to a partial vascular territory and imply assumptions for the calculation of whole heart perfusion. Because of those technical constraints, measurements can only be performed in large animal models or in humans. For studies in humans, the use of radioactive isotopes with a slow decay might be considered a health hazard. The Appendix provides some historical notes, as well as a detailed description of the coronary sinus cannulation and flow measurements.

Assessing FA Metabolism In Vivo

Radioisotope-based methods for assessment of cardiac FA uptake and oxidation in vivo are established in dog models and in humans[205–208] and are still valid. Investigators have used radiolabeled palmitate or oleate, which are the 2 most abundant FAs and are also most readily used by the heart. On the basis of these studies, 25 to 60 minutes of continuous intravenous infusion of 0.5 to 1 μCi/min of ^{14}C- or ^{3}H-FA bound to albumin is sufficient to achieve a steady state for the specific activity in arterial blood, and therefore, metabolic measurements should be performed after at least 40 minutes. Under these experimental conditions, >80% of the extracted radiolabeled FA is oxidized. Various mechanisms have been proposed to explain the phenomenon that the extraction of FAs measured by biochemical methods underestimates the extraction measured with radiolabeled FA. Assessment of FA oxidation with radioactively labeled tracers based on the specific activity of the tracer has limitations. First, it does not allow one to differentiate the fractions of radiolabeled FAs that are immediately oxidized by the heart from those first stored in the myocardial TG pool. Second, although the activity of secondarily labeled substrates such as TGs (FA incorporation) appears to be negligible during the first hour of infusion, after 2 hours TG labeling may become a contributing factor that should be included in the calculations. Thus far, no studies have specifically addressed this possibility.

Assessment of Glucose Metabolism In Vivo

Methods for direct measurement of cardiac glucose uptake and oxidation in vivo, based on radioactive and nonradioactive isotopic labeling, were first reported in human studies by Wisneski and colleagues[206–211] and are still valid. In these studies, glucose is alternatively labeled with ^{14}C, ^{3}H, and ^{13}C. A priming dose of 16 to 20 μCi followed by 0.2 to 0.3 μCi/min was used in humans and in dogs to achieve a steady state for the specific activity or enrichment in arterial blood.[209–214] For ^{13}C-glucose, the steady state for the enrichment was obtained by priming with 426 mg in a bolus, followed by an infusion of 4 mg/min. In both cases, steady state was achieved after ≈20 minutes of infusion; therefore, metabolic measurements are performed only after that time point. The protocol revealed that only 2% to 38% of extracted glucose undergoes immediate oxidation in normal hearts, depending on the arterial concentration of the competing substrate FA. Approximately 13% of it is released by the heart as labeled lactate. Secondary labeling of circulating lactate during isotope-labeled glucose infusion is not negligible. Therefore, the analysis of labeled glucose must be preceded by its separation from lactate in ion exchange columns. There are also limitations to this method, however. First, it is not possible to differentiate the fractions of labeled glucose immediately oxidized by the heart from those first stored in the myocardial glycogen pool. Second, the complex metabolic fate of more than half of the extracted labeled glucose that is neither oxidized nor released as lactate cannot be traced. Third, labeled CO_2 released in the coronary sinus derives in part from the metabolism of labeled glucose in the oxidative pentose phosphate pathway, a fraction that cannot be determined.

Assessment of Lactate Metabolism In Vivo

Direct measurements of cardiac lactate uptake and oxidation in vivo, based on radioactive and nonradioactive isotopic labeling, have been performed in dogs[215] and subsequently in humans.[209–211] In the latter studies, lactate was alternatively labeled with ^{14}C and ^{13}C. To achieve a steady state for the specific activity, a priming dose of 10 μCi was followed by 0.2 μCi/min in humans.[209–211] For ^{13}C-lactate, steady enrichment has been reached by a priming dose of 55 to 110 mg in a bolus followed by infusion at 1 to 2 mg/min in humans and dogs.[209–214] In both cases, steady state was achieved after ≈20 minutes of infusion, and therefore, metabolic measurements can only be performed after that time point. The use of isotope-labeled lactate is necessary to precisely determine the rates of lactate oxidation and release by the heart. The human heart both oxidizes and releases lactate at the same time. No major limitations of this method are known.

Directly Assessing Metabolism in the Isolated Heart Ex Vivo

The heart in vivo is, by definition, in seamless communication with its environment, whereas the well-oxygenated beating heart ex vivo can be subjected to controlled environmental conditions. A common feature of isolated perfused heart preparation is that the heart receives defined nutrients and other compounds through its native circulation. Thus, the isolated perfused heart offers the advantage of complete control of substrates, hormones, and oxygen; it also enables the examination of intrinsic myocardial properties in the absence

of external neurohormonal influences and without changes in peripheral resistance.

Langendorff Preparation

The principle of perfusing the mammalian heart retrogradely through the aorta with a physiological saline buffer solution by the method of Langendorff (1895)[216] continues to enjoy great popularity in the study of cardiac metabolism. A brief historical perspective and further details are given in the Appendix. Although many investigators have been successful in controlling and assessing left ventricular pressure development by inserting a fluid-filled latex balloon through the mitral valve,[217,218] pump work cannot be assessed. However, by changing the left ventricular end-diastolic pressure, left ventricular function can still be evaluated.

The Working Heart

In contrast to the retrogradely perfused mammalian heart, the isolated working heart allows the investigator to assess pressure volume work, myocardial oxygen consumption, and rates of substrate utilization simultaneously. The principle of the working heart preparation antegradely perfused via the left atrium was first developed by Otto Frank[219] and later adapted for the rodent heart,[30,31] including the mouse heart.[220] The isolated, perfused working heart remains the gold standard for quantitative assessment of the dynamics of cardiac metabolism because it permits stoichiometric assessment of energy conversion by the heart under near physiological loading and working conditions. Please refer to the Appendix for details.

Metabolism of Isolated Cardiac Cells and Cell Lines

The study of cardiomyocytes in vitro has contributed much to the understanding of the cellular and molecular mechanisms underlying the regulation of cardiac metabolism. Isolated cardiomyocytes and selected muscle cell lines provide a model system that is devoid of nonmyocytes. In addition, isolated cardiomyocytes offer the following advantages: (1) full control of the environment, in particular the concentrations of substrates, hormones, cytokines, etc, with no interference of gradients because of the capillary wall and fluctuations in coronary flow; (2) the ability to select cells from different areas of the heart; (3) the application of gene transfer to upregulate or downregulate specific genes of interest; and (4) fewer experimental animals are required, which is an advantage (eg, in pharmacological studies).[221–223]

Cardiomyocytes From Rat and Mouse

As postmitotic cells, adult cardiomyocytes do not proliferate. Therefore, primary cardiomyocytes are isolated and kept in culture with appropriate media to preserve their in vivo properties in an optimal environment in vitro. During culture, the incubation media must mimic the in vivo environment as much as possible, including a near-physiological mixture of metabolic substrates, to avoid adaptation of the cells and acquisition of altered characteristics.[223] The composition of the incubation media must be exactly known. Current methods allow the reproducible isolation of high-quality adult cardiomyocytes that remain alive but show no spontaneous contraction in media of physiological Ca^{2+} concentration and temperature; that is, Ca^{2+}-tolerant cells.[223,224] Isolation of ventricular myocytes from adult rat or mouse is a multistep process that involves retrograde perfusion through the aorta with enzyme-containing solutions (Langendorff mode), followed by gentle trituration of the tissue and repeated sedimentation of cells to separate viable rod-shaped cardiomyocytes from the rounded dead myocytes, which float. The yield and quality of the cells obtained depend on a number of variables, in particular the composition and purity of the collagenase lot and the composition of media to render the cells Ca^{2+} tolerant.[221,223,225] In general, these parameters are more critical with the isolation of cardiomyocytes from mouse than from rat.[224] Reported yields of isolated cells typically are ≈50% (mouse) and ≈70% (rat), which indicates that selection of cardiomyocyte subpopulations is not a major concern.

Isolation of embryonic or neonatal cardiomyocytes is easier than the isolation of adult cardiomyocytes and consists of a 2-step procedure with enzyme digestion and mechanical agitation of the ventricular tissue followed by purification of the cardiomyocyte population.[223] Importantly, as in the in vivo heart, the metabolic characteristics of neonatal and fully differentiated adult cardiomyocytes differ considerably (eg, rates of lipid metabolism are markedly lower in neonatal cells), so that for metabolic studies, neonatal cardiomyocytes cannot be compared directly with adult cells.

Electrical field stimulation results in rhythmic contractions of the isolated cardiomyocytes in suspension and enhances oxygen consumption and substrate uptake and oxidation in linear dependence on the rate of stimulation.[226,227] The metabolic rates in electrical field–stimulated cardiomyocytes are lower than those measured in the intact beating heart.[227,228]

Certain hormones, notably insulin, also increase substrate uptake by cardiomyocytes. Isolated cardiomyocytes are therefore used as a model to examine the metabolic effects of environmental factors (hormones, cytokines, pharmacological agents) independent from contractions. For example, research using isolated cardiomyocytes has established the signal transduction pathways and molecular mechanisms involved in the regulation of GLUT4-mediated glucose uptake and CD36-mediated FA uptake and utilization in the heart.[229]

Culturing isolated cardiomyocytes also allows interventions such as downregulation or (over)expression of specific genes (by gene transfer), as well as subjecting the cells to altered environmental conditions, including hypoxia. Cardiomyocytes cultured in monolayers are also used to study mechanoelectric properties of the heart,[230] but not for metabolic studies. Methods that preserve as closely as possible the in vivo integrity and function of the myocytes during 1 to 2 weeks of culture are still undergoing improvements.[223,231,232] Nevertheless, with current culture methods, specific morphological and functional alterations do occur. Myocytes become more rounded as opposed to square-edged, rod-shaped appearance, and show a decreased T-tubule density, as well as specific changes in ion channels and ion currents. Therefore, testing myocytes for culture-related changes in the parameter to be measured is a necessary control for any study protocol.

Apart from obtaining cardiomyocytes from rodent disease models, metabolic changes in pathophysiological conditions can be examined in cardiomyocytes that acquire such conditions in vitro. For instance, chronic exposure (48 hours) of primary cardiomyocytes from healthy rodents to a medium with a high palmitate concentration elicits excess lipid storage followed by insulin resistance of the cells.[233] This established in vitro model of insulin-resistant cardiomyocytes allows for detailed studies on the pathogenesis of FA-induced cardiac insulin resistance and the rapid screening of putative therapeutic interventions.[234]

Lastly, methods commonly applied to assess metabolic rates in isolated cardiomyocytes include measurement of the production of CO_2 (trapped in hyamine hydroxide or any other appropriate base solution) and acid-soluble intermediates from radiolabeled or deuterated substrates[228,234] and measurement of mitochondrial oxygen consumption by permeabilized cells.[235]

Rodent Cell Lines

A number of immortal cell lines have been introduced as models for cardiomyocytes. These cell lines are all of atrial origin and include H9c2 cells[236] and HL-1 cells.[237]

H9c2 cells are of limited use because they lack many adult cardiomyocyte characteristics. For instance, H9c2 cells have a low mitochondrial content and a very low capacity for FA oxidation. HL-1 cells have been characterized extensively.[237,238] In culture, HL-1 cells remain similar to primary cardiomyocytes with respect to their signaling and transcriptional and metabolic regulation. HL-1 cells can be electrically paced to contract (in a noncoordinated manner as the contractile elements are dispersed), genetic interventions can be readily applied, and the cells have been used to address pathological conditions such as hypoxia, hyperglycemia-hyperinsulinemia, apoptosis, and ischemia-reperfusion.[237] HL-1 cells can also be made insulin resistant, thereby responding in a manner similar to primary cardiomyocytes.[239,240] Taken together, HL-1 atrial cardiomyocytes can be considered a useful model system to study metabolic and other aspects of cardiac physiology and pathophysiology at the cellular level because they demonstrate characteristics of differentiated cardiomyocytes.[238]

Cardiomyocytes of Human Origin

Various sources of human cardiomyocytes are available; that is, ventricular cells isolated from human cardiac biopsy samples, a cell line (AC16) derived from adult human ventricular cardiomyocytes, and cardiomyocytes derived from human induced pluripotent stem cells (iPSCs) for metabolic studies. Protocols for isolating viable myocytes from small surgical samples exist and involve enzymatic digestion of the tissue fragments excised during elective surgical procedures followed by purification, resuspension, and Ca^{2+} readaptation of the cells.[241,242] These human myocytes are mostly used to characterize specific functional (electrophysiological and Ca^{2+} handling) abnormalities in patients. The small yield of cells (because of the limited size of the biopsy specimen) limits their use for assessment of metabolic activity until a highly sensitive single-cell technique becomes available. The AC16 cell line was generated by fusion of primary cells from human ventricular tissue with SV40-transformed human fibroblasts.[243] AC16 cells have retained the nuclear and mitochondrial DNA of primary cardiomyocytes and have fully functional metabolic characteristics, which makes them useful for metabolic studies.

iPSCs are emerging as a unique and promising source to obtain cardiomyocytes for both animal- and patient-derived studies ("personalized medicine").[244,245] iPSC-derived cardiomyocytes recapitulate disease phenotypes from patients with monogenic cardiovascular disorders and are now being deployed to provide insights regarding drug efficiency and toxicity.[245] Regarding metabolic phenotyping, the unique metabolic features of stem cells, cardiomyocytes, and their progenitors have been reviewed.[246] Notably, differentiation of iPSCs into cardiomyocytes is accompanied by a shift from anaerobic glycolysis to mitochondrial respiration, just as occurs in vivo during perinatal cardiac development. The acquisition of an FA metabolic signature during this transition is important so the high-energy demands of differentiated somatic cardiomyocytes are met. As a result, the routine availability of human iPSCs and of tools for their controlled differentiation into cardiomyocytes offers unique opportunities to identify critical metabolic processes in the transition between proliferating and differentiated somatic cells and vice versa. For instance, lactate-enriched culture media are preferred for enriching cardiomyocytes from iPSCs, because such media are well tolerated by cardiomyocytes but not by other differentiated cells.[247] Such an approach has been proposed to obviate the need for genetic manipulation in the generation of stem-cell derived somatic cells for therapeutic applications.[246] Furthermore, the inherent flexibility in energy substrate metabolism underlies the cells' ability to prioritize certain metabolic pathways in support of stage-specific demands.[248] The combination of a massive suspension culture system with metabolic selection holds promise as an effective and practical approach to purify and enrich the large numbers of cardiomyocytes found in iPSCs.[248]

In summary, cardiomyocytes in suspension are a valuable tool for assessing cardiac metabolism at the cellular and molecular level, thereby providing insights representative of the intact heart. With the current availability of several cardiomyocyte sources (ie, cells isolated from cardiac tissue, permanent cell lines, and human iPSC-derived cardiomyocytes), the significance of these model systems for assessing cardiac metabolism is expected to increase.

Mitochondria (Including ROS)

Isolated Mitochondria and Cellular Bioenergetics

The canonical function of mitochondria is the generation of ATP by oxidative phosphorylation of ADP. Mitochondrial dysfunction contributes to a broad range of pathologies, particularly those involving the cardiovascular system, with compelling evidence implicating changed bioenergetics in both the vessel wall and the cardiomyocyte.[249–251] Evaluation of mitochondrial function has moved beyond simply isolating the organelle by disruptive techniques and measuring parameters such as respiratory control ratio and state 3/4 respiration. The contemporary approach to bioenergetics is guided by

the mitochondrial quality control paradigm.[252–255] This model centers on the dynamic aspects of mitochondrial function that have broadened the biological end points relevant to analysis of cellular bioenergetics. The focus is now on how cells under metabolic, genetic, or environmental stress maintain the quality of the mitochondrial population.

Mitochondrial quality is maintained through a well-regulated balance between mitochondrial biogenesis, the maintenance of bioenergetic reserve capacity, fission, fusion, and mitophagy.[254,256] Mitophagy is particularly important because it removes the damaged mitochondria that might otherwise contribute to a progressive bioenergetic dysfunction and programmed cell death.[257]

In the rapidly evolving interface between redox biology, bioenergetics, and CVD, investigators are beginning to question some long-held assumptions. For example, instead of mitochondrial superoxide and hydrogen peroxide production as the major source of oxidative stress in the cell, an intricate "retrograde" signaling pathway that coordinates mitochondrial function with the demands of the cell through regulation of transcriptional events in the nucleus has been proposed.[258] Importantly, new animal models are revealing the impact of mitochondrial genetics in controlling inflammation through retrograde signaling pathways.[259] These animal models aid in translating the emerging concepts of redox biology and bioenergetics as we strive to understand how the pro-oxidant and proinflammatory environment associated with the sedentary western lifestyle contributes to increased risk for CVD.

A gateway question often posed at an early stage in both basic and translational bioenergetics research is, how is mitochondrial function changed in response to an experimental pharmacologic/genetic model or clinical pathology? Classically, this has been addressed through experiments with isolated mitochondria performed in the context of established protocols in which oxidative phosphorylation or activity of candidate metabolic enzymes was measured. Typically, the outputs from such experiments are the rates of state 3 and 4 respiration and the ratio of these 2 rates (ie, the respiratory control ratio). Mitochondrial damage is then defined as a deviation of these parameters in an experimental or disease group relative to controls. This information remains of value, but with the advent of high-throughput respirometry, the strategy for assessing mitochondrial function can now be extended to encompass metabolomics, energy-rich phosphates, and cellular bioenergetics.

The first step in measuring mitochondrial function is frequently the evaluation of cellular bioenergetics, which we define as the field in biochemistry concerned with the flow of energy through living systems. The rationale for beginning the experimental approach at the level of the cell (rather than isolated mitochondria) is that isolated mitochondria are not connected to the rest of the cell, lack the signaling to control metabolism, and use saturating, nonphysiological concentrations of substrates and ADP. Methods, design of experiments, and interpretation of data are all described in the literature.[254,260] The basic experimental paradigm is known as the "mitochondrial stress test," which results in 6 cellular respiration parameters obtained from the sequential addition of inhibitors of oxidative phosphorylation.[254]

Of these, the parameters known as reserve capacity and ATP-linked respiration are of particular interest because they allow some estimate of the cells' energetic demand under a given condition and the extent to which additional energetic demand can be met.[254] In general, a decreased reserve capacity is associated with increased susceptibility to oxidative stress.[261–263] An interesting aspect of cellular bioenergetics is that the impact of different fuels on bioenergetic function can also be determined in a cellular context, and by using the simultaneous measurement of pH, information about the activity of the Krebs cycle (in the mitochondrial matrix) and glycolysis (in the cytosol) can also be obtained.

Next, mitochondrial function can be determined in a parallel experiment with the same cells by permeabilization of the plasma membrane with cholesterol-specific chelating detergents, such as saponin or the glycoside digitonin,[264] or proteins, which allows addition of exogenous substrates and ADP to measure classic mitochondrial parameters.[265] This approach is based on the fact that the inner mitochondrial membrane contains very little cholesterol compared with the plasma membrane. This is a preferred approach over isolating mitochondria because it avoids artifacts related to biasing the mitochondrial populations during isolation after tissue separation. It is also more sensitive. In addition, because of the interaction of mitochondria with the cytoskeleton in vascular and endothelial cells, preparation of functioning mitochondria is extremely difficult. Thus, permeabilized cells are the better option.

The techniques for the preparation and characterization of different mitochondrial populations from heart samples are also well established.[266] Measurements in freshly isolated subsarcolemmal and interfibrillar mitochondria[267,268] or permeabilized cells can then be used in conjunction with the cellular bioenergetics to identify defects in specific segments of the oxidative phosphorylation pathway. Mitochondrial subpopulations are affected by cardiac pathologies such as concentric hypertrophy, heart failure, ischemia/reperfusion, or systemic metabolic dysregulation (obesity and diabetes mellitus). An extensive discussion on this topic appeared recently.[267] In the absence of oxidative phosphorylation, changes in cellular bioenergetics may be observed because of changes in substrate supply. In this case, additional measures are required (eg, substrate uptake assessment or a targeted metabolomics approach).

Change in mitochondrial membrane potential is a frequently used method, although it is not always appreciated that the response of the dyes to membrane potential follows a logarithmic, nonlinear relationship. This means that small changes in signal can represent large changes in mitochondrial membrane potential, which can be revealed by use of appropriate controls.[269] New optical mapping techniques hold the promise to assess mitochondrial membrane potential and depolarization in the intact heart with potentiometric dyes.[270]

Mitochondrial Superoxide and Hydrogen Peroxide Production

Over the past few years, new perspectives on mitochondrial superoxide and hydrogen peroxide have evolved. It is quite clear that relative to enzyme systems designed solely

to generate superoxide, such as the NADPH oxidases,[271,272] mitochondria are also a source of ROS in the cell. The small amounts of superoxide generated from different segments of redox centers in the mitochondrion are linked to metabolism and play a role in cell signaling.[258] With isolated mitochondria, measurement of hydrogen peroxide can be both specific and quantitative, with sensitive assays that couple hydrogen peroxide production to fluorometric or spectrophotometric oxidation of dyes using peroxidases.[273] The technical problem lies in determining what metabolic condition should be applied to the isolated mitochondria to make these measurements relevant physiologically. Recent studies outline how this can be achieved.[273] In-cell measurements have the advantage that the physiological relevance is less in question, but then the problem is a loss of both specificity and quantitation. New probes that use protein-based specific redox sensors targeted to particular submitochondrial compartments are becoming available.[274] Interestingly, it has already been shown that the redox environments of the matrix and intermembrane space are very different. An alternative approach is to use a surrogate for changes in intramitochondrial redox status, such as the oxidation status of protein thiols.[275] The oxidation of protein thiols can be measured with a high degree of precision, and protein thiols are both abundant and critical for metabolic and signaling function in the mitochondria.[276] Targeting redox active molecules to the mitochondrion is an effective strategy to probe mechanisms of redox signaling, but appropriate controls are needed to avoid artifacts caused by the nonspecific effects of the targeting moiety.[277,278]

Mitochondrial DNA is particularly vulnerable to low levels of oxidative damage and can be measured easily in isolated mitochondria, cells, and tissues.[279] Loss of intact mitochondrial DNA may become limiting in the biogenesis process required to replenish the damaged mitochondria removed by mitophagy and may ultimately contribute to the deteriorating quality of the mitochondrial population.[254,280]

Fission-Fusion, Mitochondrial Ca^{2+}, Mitophagy

In a cellular context, mitochondria are networked to a reticulum, and the regulation of these networks is critical in normal cell division and the process of mitophagy. Networks are highly dynamic and regulated by a critical balance between fission and fusion.[281–283] There is evidence for a role of mitofusin 2 in tethering mitochondria to the sarcoendoplasmic reticulum in cardiac myocytes.[284] Please refer to the Appendix for the concept of mitochondrial Ca^{2+} microdomains and Ca^{2+} regulation of mitochondrial function.[285] The expression of green fluorescent proteins in the mitochondria allows for the dynamic monitoring of mitochondrial networks under normal and pathological conditions.[286] The same approach can be used to determine the activity of the mitophagy pathway, which under many conditions, including oxidative stress, is protective.[287] Because antibodies have become available for many of the key proteins involved in autophagy and mitophagy, it is relatively straightforward to determine the underlying mechanisms that lead to a change in the status of the mitophagy pathway.[288] Further discussion on translational bioenergetics and mitochondria as a regulator of cell death is included in the Appendix. The cardiac mitochondrial interactome has recently been reviewed in its full context.[289]

Proteomics and PTMs

Proteomics and Cardiac Metabolism

Proteomic technology is reinforcing the power of cardiac metabolism to induce posttranslational changes through covalent modifications of functional proteins. Over the past 20 years, proteomics has provided unexpected insights that have changed our thinking about the complexity of the proteome disease process, identified unexpected functional end effectors involved in signaling pathways, and shown that mRNA and protein concentrations do not necessarily correlate but that protein dynamics play an important role. This is true in all aspects of metabolism, including the mitochondria, under physiological and pathophysiological conditions. An extensive background section on proteomics is found in the Appendix. A few examples of the importance of proteomic analyses are given here. As mentioned previously, intermediary metabolism is subject to circadian regulation, which ensures that there is an appropriate temporal organization of metabolism and ensures adequate energy is provided to the various subproteomes of the heart. Proteomic analysis of cardiomyocyte clock mutant mice has revealed that the cardiac proteome is significantly altered in cardiomyocyte clock mutant hearts, especially enzymes that downregulate vital metabolic pathways and are in tune with the contractile myofilament subproteome's loss of time-of-day–dependent maximal calcium-dependent ATP consumption.[62] With exercise-related metabolic demands in rats after 54 weeks of moderate treadmill exercise, proteomic analysis clearly shows modulation in the abundance or phosphorylation status of proteins involved in the generation of precursor metabolites and in cellular respiration.[290] In fact, there was an increased concentration of RAF and p38 mitogen-activated protein kinases within mitochondria, which suggests that the heart mitochondria reprogram both by changes in protein quantity and by several signaling pathways that in turn regulate metabolic pathways. Examples of metabolically relevant proteome changes in human heart failure are given in the Appendix.

The proteome is complex, but so too are single proteins. For example, a protein can have multiple splice variants. More than 1 amino acid can be modified in a single protein, and a single amino acid residue can be modified by more than 1 type of PTM. In fact, different PTMs can compete for the same amino acid residue. Within the human cardiac 20S proteasomes, there are 63 Lys that are ubiquitinated lysines and 65 acetylated Lys, with approximately two thirds (or 39) of them shared.[291] Desmin, an intermediate filament protein involved in the alignment of mitochondria with the nucleus, myofilament, and the cell membrane, undergoes glycogen synthase kinase-3β phosphorylation in many models of heart failure, which alters its susceptibility to selective proteolysis and subsequent amyloid formation.[292] The extent of phosphorylation and proteolysis is decreased with cardiac resynchronization therapy, which also reveals a reduction of amyloid-like oligomer deposits. The link between phosphorylation and proteolysis

in heart failure and its reversal toward control levels with cardiac resynchronization therapy is also observed for the F_1F_0 ATP synthase β-subunit, which produces the majority of the ATP in the cell.[293] Further proteomics analysis also revealed that regulation of this important enzyme is redox sensitive and that oxidation of a particular Cys residue in the α-subunit can completely (and dramatically) reduce the enzymatic activity of this important protein complex.[294]

Fine mapping of PTMs and site-specific quantitation of specific residues are possible only through the use of PTM-specific enrichment and tagging and the use of MS.[295,296] Not surprisingly, there are broad functional changes with cardiac resynchronization therapy, including contractility. In fact, phosphoproteomic analysis of the heart has identified many novel M and Z line proteins and not the traditional regulatory thick filament proteins.[297] This may be considered paradigm changing in the realm of metabolism, too,[298] because it opens up a new way of viewing pathophysiology and coordination (or dys-coordination) of the broad cell response.

Proteomics also often reveals unexpected findings in metabolism. For example, MS first showed phosphorylation of the β-subunit of F_1F_0 ATP synthase.[299] This was a paradigm change, because this phosphorylation occurs within an hour of the administration of a preconditioning agent, adenosine. For a protein located within the inner mitochondrial membrane to be phosphorylated strongly suggests the presence of a number of kinases within the mitochondria and not just associated with the outer membrane.

Proteomic approaches are used to analyze the formation of superassembly/supercomplex formation of the F_1F_0 ATP synthase superassembly in mitochondria.[300] With the important development of selected reaction monitoring assays for an increasing number of mitochondrial proteins and enzymes involved in metabolism, including, in some cases, their phosphorylated forms, this opens up the possibility of precisely monitoring proteome changes in the heart under diverse conditions of metabolic stress.[301]

The field of proteomics continues to develop improved reagents for assessment of PTMs. This includes oxidative PTMs, such as those induced by reactive nitrogen species, such as *S*-nitrosylation (also known as *S*-nitrosation), and ROS, including sulfenic acid (SOH, and the irreversible sufinic and sulfonic acid), which target cysteine or carbamylation and nitration, all of which can modify other amino acid residues. The ability to identify and quantify oxidative PTMs is necessary to determine the mitochondrial roles that occur beyond metabolism, in which mitochondria act as significant sources of signaling molecules, including ROS. A number of new cysteine thiol-labeling strategies have been developed and have exploited MS. These include proteomic investigations on the effect of cardiac protection,[302,303] metabolic syndrome,[304] hypoxia,[305] and doxorubicin treatment.[306] In all cases, there is extensive modification of Cys or other residues in key proteins involved in the regulation of energy substrate flux, glycolysis, β-oxidation, the Krebs cycle, and oxidative phosphorylation. However, the issue is more complex. A recent proteomic study has shown that nitric oxide synthase and L-arginine are also regulated by PTMs, specifically via the kinase ROCK, which results in the oxidized low-density lipoprotein–triggered translocation of arginase 2 from the mitochondria to the cytosol in human aortic endothelial cells and in murine aortic intima.[307]

As discussed previously, metabolites themselves can provide both signals and building blocks for growth or destruction of proteins through protein quality control, autophagy, and the ubiquitin proteasome pathway. Moreover, mitochondria can rejuvenate by undergoing fission and fusion. The mitochondrial specific deacetylase, SIRT3, deacetylates the mitochondrial fusion protein OPA1, increasing its GTPase activity. MS has unambiguously identified Lys 926 and 931 as the sites modified in OPA1, which enabled subsequent experiments to prove that SIRT3-dependent activation of OPA1 preserved the mitochondrial network and correlated to enhance cardioprotection.[308] In fact, acetylation of the cardiac proteome is extensive, as shown in global acetylation studies.[309] However, there are also methods for performing selected reaction monitoring on specific acetylated proteins (eg, acetylation succinate dehydrogenase A).[310]

Lastly, proteomics has proven that relatively subtle perturbations in metabolic fluxes can rapidly impact protein function, localization, and stability through reversible PTMs; *S*-nitrosylation, acetylation, and O-GlcNAcylation are metabolism-derived PTMs that modulate cardiomyocyte form and function. As discussed previously, methods for quantitative assessments of protein kinetics have been challenging. Deuterium oxide (^2H_2O) labeling and high-resolution MS have been used to systematically interrogate in vivo protein turnover of ≈3000 proteins.[171] In an example from a mouse model of cardiac remodeling, widespread kinetic regulations could be found across most of the cell, including calcium signaling, metabolism, proteostasis, and mitochondrial dynamics. Once more, proteomic data support the concept that the cardiac myocyte adapts to change broadly across the proteome, with metabolic and mitochondrial proteins most prominently displayed.

Metabolomics

Metabolomics as a Discovery Tool

The identification of multiple metabolites by metabolomic techniques has renewed the interest in metabolism across biology and cardiovascular research. Similar to other –omic approaches, metabolomics,[311] which is also referred to as metabonomics,[312] aims to identify a wide array of molecules, specifically, the small-molecule chemicals involved in the metabolism of a cell, tissue, biofluid, or organism. Metabolomics is of particular use as a functional tool to identify changes associated with a given genetic, pharmaceutical, or pathophysiological stimulus. Although there are a number of similarities between metabolomics, transcriptomics, and proteomics (Figure 2), the field of metabolomics has its own unique challenges. Both mRNA and proteins consist of a rather limited number of building blocks (nucleotides and amino acids, respectively), but this cannot be said of metabolites, which range in polarities from the highly polar to highly lipophilic compounds. In addition, metabolites range in concentration in most cells from millimolar (10^{-3} mol/L) to femtomolar (10^{-15} mol/L) and below. Thus, no single analytical

tool is able to cover the entire metabolome. This limitation has not hindered its application in medical sciences, for which metabolomics is increasingly used to follow a range of cardiovascular disorders, as well as functional genomics of heart function. Comprehensive reviews are in the literature.[94,313,314]

Extraction Procedures for Metabolomic Analyses

As in all metabolic studies, sample preparation and the extraction protocol are the critical first steps in the analytical workflow. In terms of procurement, considerations should be made regarding choice of anesthesia, rapid tissue isolation, and appropriate storage. The extraction protocol must provide adequate recovery of a given metabolite, as well as quench metabolism so that reactive intermediates are not degraded during isolation of biochemicals from cells, tissue, or body fluid. This is a particular challenge in metabolomics, in which the aim is to solubilize a wide range of metabolites in such a manner so as not to bias subsequent analyses. One commonly used extraction procedure uses a combination of chloroform and methanol to extract both aqueous and lipophilic metabolites[315]; however, such biphasic protocols are not always appropriate. For example, when carnitine derivatives are analyzed, free and shorter carnitines partition into the aqueous fraction, whereas the longer chain carnitines are found in the lipophilic fraction. In such situations, it might be better to opt for a single extraction, such as ice-cold methanol, which, although not optimized for any individual carnitine species, provides good recovery across the range of carnitine derivatives. A common approach is to use the statistical tool design of experiments to optimize extraction protocols.[316]

Analytical Tools for Metabolomic Analyses

The 2 most common analytical tools used in cardiac metabolomics are high-resolution NMR spectroscopy and MS. NMR spectroscopy is inherently quantitative and the process highly reproducible, because nothing is degraded during the analytical process. However, because the nuclear transitions are comparable in energy to thermal energy at room temperature, there is a low signal to noise ratio, and the approach largely detects metabolites in a range between 100 μmol/L and 100 mmol/L.

The second method, MS, relies on initial ionization of a molecule with either a positive or negative charge (referred to as positive or negative mode) and then acceleration of the ion by use of magnetic, electrical, or both types of fields. Such instruments are capable of detecting femtomoles of material, providing a highly sensitive approach for measuring the metabolome. When metabolites are infused directly into a mass spectrometer, the method is referred to as direct-infusion MS.

Because of the complexity of the metabolome and the sensitivity of MS, some form of separation before the MS is often required. The 2 most common approaches are GCMS and LCMS. In GCMS, volatile metabolites are introduced in the gas phase (referred to as the mobile phase) into a column that contains a solid-phase material (referred to as the stationary phase), which encourages absorption of these volatile metabolites. The temperature of the column is then raised, and at some stage, the metabolites re-enter the gas phase and pass into the mass spectrometer. This method is widely used to examine both total FAs (as FA methyl esters) and aqueous metabolites after derivatization in both cases.[317] In liquid chromatography, the mixture is introduced in solution (mobile phase) onto a column with a solid (stationary) phase that encourages interactions between the material and the metabolites. The most commonly used approach is referred to as reverse-phase chromatography, in which the metabolites are deposited on the material in a solvent that is predominantly aqueous in nature, and metabolites are removed from the column as the mobile phase becomes more organic in nature. A complication of this approach is that the solvent must be removed during the ionization process, for which electrospray ionization is most commonly used. LCMS is used to analyze a wide range of aqueous metabolites and lipid species.

Although mass spectrometers can be operated in a full-discovery mode, acquiring mass/charge information in an unbiased manner, they can also be configured to assay a selected number metabolites in a targeted manner. For targeted approaches, the user focuses on a predefined set of metabolites (typically several dozen to hundreds) of known identity for detection and quantitation. Tandem MS has proven to be an invaluable tool for such approaches, in particular using a triple quadrupole mass spectrometer. Here, the investigator can selectively monitor for specific parent/daughter ion combinations that correspond to known metabolites, as confirmed empirically by analyses of commercial standards. Although the targeted approach generates a narrower view of the metabolome, biased toward a predefined set of analytes, researchers can have more confidence in the end results because they know what is giving rise to the signals "up front." By contrast, unambiguous identification of a peak identified in an unbiased profiling experiment can take days to months. Mass spectrometers are also more sensitive when operated in a targeted fashion, acquiring data only for specific mass to charges. Although the metabolite identification is known a priori, its association with a given phenotype may still be novel. A discussion of biomarker studies in humans is in the Appendix.

From Association to Causation: Integrating Metabolomic and Genetic Findings

The integration of genetic and metabolomic data in humans represents an exciting strategy to establish a causal link between metabolite biomarkers and disease. For a biomarker that has a causal role, the expected random distribution in a population of a polymorphism that determines high or low biomarker concentrations would be skewed in individuals depending on their disease status. The association between a disease or a metabolite and a genetic polymorphism that mimics the biological link between a proposed exposure and disease is not confounded by the reverse causation that affects many epidemiological observational studies. Data from mendelian randomization studies are accumulating for several biomarkers. Analyses demonstrating that single-nucleotide polymorphisms that modulate plasma low-density lipoprotein cholesterol concentrations are independently associated with incident CVD are consistent with the known causal role low-density lipoprotein cholesterol plays in atherogenesis.[318]

By contrast, genetic loci associated with plasma C-reactive protein concentrations (including in the CRP locus) have no association with coronary heart disease, which argues against a causal association between C-reactive protein and CVD.[319]

Recent work has begun to explore the genetic determinants of plasma metabolite levels in large human cohorts.[320,321] Many of the findings in these studies highlight locus-metabolite associations with strong biological plausibility. For example, the locus of interest includes a gene encoding a protein directly responsible for the metabolism or transport of the given metabolite. However, many other locus-metabolite associations have been identified in these studies without a clear biochemical relationship with the given metabolite, stimulating further investigations, particularly when the metabolite of interest is associated with a disease phenotype. An alternative approach that can be taken is to begin with disease-associated single-nucleotide polymorphisms and then integrate the NMR or MS data to begin to understand the metabolic consequences of the human variant of interest. As ongoing studies further delineate the genetic determinants of plasma metabolite profiles, efforts to triangulate gene-metabolite-disease associations will provide insight into whether and how metabolite markers contribute to disease pathogenesis, studies that can be further buttressed by pathway modulation in animal models.

Genetic Models for Cardiac Metabolism

Advances in genetic engineering have resulted in an exponential increase in the development of genetic models that have been used to study or address important questions regarding cardiac metabolic homeostasis. The overwhelming majority of these models have been developed in the mouse, with a smaller number in rats. Although many of the pathways that direct myocardial substrate utilization have long been described, genetic studies in vivo have not only confirmed putative roles for key metabolic enzymes or pathways but have also challenged some paradigms, such as the direct regulation of myocardial FA oxidation by malonyl-CoA concentrations.[136,322] They have also at times provided surprising insights regarding previously unknown functions,[323] the existence of overlapping or redundant pathways,[324–326] or novel interactions between metabolic pathways and other aspects of cardiac function such as excitation-contraction coupling.[118,327] In addition to models in which specific metabolic enzymes or transporters are perturbed, genetic models have also provided important insights into regulatory pathways, with profound effects on metabolic pathways within the myocardium. Recent advances in gene therapy methods have also enabled acute changes leading to functional gain and loss of metabolic enzymes in otherwise naïve animal models.[136,328,329] This approach averts developmental adaptations to metabolic changes while elucidating short-term responses when the expression of enzymes associated with metabolic processes is altered.[197]

In the broadest sense, 2 types of genetic models have been established. They are gain-of-function and loss-of-function mutations. Most gain-of-function models have been generated by overexpressing the gene of interest specifically in cardiomyocytes downstream of a promoter that restricts expression to cardiomyocytes, commonly the α-myosin heavy chain promoter (α-MHC or MYH6). The earliest (and still commonly used) approaches resulted in lifelong constitutive overexpression of the protein of interest, and studies were performed at various stages throughout the animal's life.

There are caveats that should be considered in the examination of transgenic overexpression models. First, random insertions of transgenes with varying copy numbers can result in various degrees of overexpression. There are many examples in the literature of high-level transgene overexpression having nonspecific toxicity, leading to left ventricular dysfunction, which can have independent effects on cardiac metabolism. Thus, it is important to examine multiple lines with various levels of overexpression (low to high) to determine whether the gene dose correlates with putative physiological function of the overexpressed protein. A second caveat of this approach is lifelong adaptations that can confound phenotypic analysis, given that promoters used to drive cardiomyocyte transgene expression may express as early as embryonic day 10.5, as in the case of MYH6. A number of recent refinements now allow the conditional temporal expression of transgenes within cardiomyocytes by coupling transgene expression with the cardiomyocyte-restricted expression of a transcriptional regulator whose activity is coupled to the presence or absence of a drug such as doxycycline.[330] This approach has in some instances provided an important distinction between the impact of transgene expression from birth versus transgene induction in the adult heart, as exemplified in the phenotypic differences between mice with lifelong overexpression versus inducible expression of the GLUT1 glucose transporter.[331,332]

Initial loss-of-function studies examined animals with germline loss of alleles of interest, and although many of these models shed light on cardiac metabolism, systemic effects of the gene deletion might confound the interpretations of changes observed within the heart. It may be considered that modeling of genetic human disorders such as inborn errors of metabolism might be best suited to germline deletion or insertion mutagenesis (knock in) strategies. Although true, most human disorders associated with alterations in cardiac metabolism do not have a genetic basis, and differences in whole-animal metabolic adaptations in humans versus rodents might lead to divergent systemic responses to germline mutagenesis. As such, cell-type or temporal gene deletions are now predominantly used, particularly given their ability to generate less ambiguous mechanistic insights. Thus, widespread adoption of Cre-mediated gene targeting has allowed for the generation of mice with cardiomyocyte-restricted knock out of many metabolic genes of interest. Most studies have used MYH6-Cre to drive cardiomyocyte-restricted gene deletions, and as is the case with transgenic studies, these models (that have been for the most part very informative) are also confounded by potential adaptations to lifelong gene deletion within cardiomyocytes. Recent refinements such as tamoxifen- or doxycycline-regulated and cardiomyocyte-restricted Cre drivers have enabled analyses of short-term disruption of relevant pathways within adult cardiomyocytes and in some cases have highlighted important differences based on the timing of gene deletion.[325,333–335]

An important consideration in all of these approaches is the utilization of appropriate controls. Specifically, given the important effects of genetic background on many mouse

phenotypes, it becomes critically important at a minimum to analyze littermate controls or preferably to backcross mutants and controls to a uniform genetic background, classically done by backcrossing >7 generations, although this process can be accelerated by generating speed congenic strains, which can be facilitated by genotyping and screening offspring for genetic polymorphisms that are specific for a given background strain. Second, the use of drugs such as doxycycline or tamoxifen might have independent effects on cardiac mitochondria or cardiac metabolism.[336] Therefore, controls should include drug-treated cohorts without floxed alleles or examination of Cre-expressing lines with wild-type alleles to establish potential background effects that could confound phenotypic analyses.

Mouse models with targeted mutations (overexpression or gene deletions) of most of the components of these classical metabolic pathways are summarized in the Appendix (Table A3). This traditional view of the role of the creatine kinase system has been challenged recently.[337] These canonical pathways are subjected to additional regulation by the generation of allosteric modulators or by cell surface– or nuclear receptor–based signal transduction pathways, or by genomic regulation by transcriptional coactivators or microRNAs. Many of these pathways have also been genetically modified in mouse mutant models and are summarized in the Appendix (Table A4). Space constraints do not allow a detailed description of each of these models, but Table A4 provides a brief highlight of the critical findings of these studies. Instead, some general comments are now provided to give the reader a framework for more in-depth exploration of this large body of work.

In some models, altered expression of a transporter, enzyme, or regulator of cardiac metabolism may yield a striking phenotype in nonstressed hearts. In other scenarios, phenotypes only become apparent when the heart is subjected to additional stressors such as hemodynamic stress (eg, pressure-overload hypertrophy, coronary ligation) or metabolic stress (eg, diabetes mellitus or high-fat diets). Genetic models have generally supported the concept that increased glucose utilization in the context of ischemia/reperfusion is beneficial, although important exceptions have been noted.[338] Moreover, despite the observation that increased glucose utilization has been viewed as an adaptive response that may limit the transition from compensated left ventricular hypertrophy to heart failure, loss of substrate supply irrespective of carbon source (glucose or FA) appears to be critical in this transition. Indeed, increasing mitochondrial FA oxidation also preserves or maintains contractile function in pressure-overload left ventricular hypertrophy.[106,339,340] Second, some models have revealed the extraordinary adaptability of the heart to limitations in substrate preference with minimal long-term effects, even in response to stress, whereas others have provided clear evidence of metabolic inflexibility. In general terms, adaptability of the heart correlates with duration of transgene expression or gene knockout, such as with promoters that drive altered gene expression early in development. In contrast, temporal deletion or overexpression of genes in the adult hearts tends to induce changes that, in the time frame of the studies performed, are less well tolerated. The studies summarized in Tables A3 and A4 have also revealed redundant or overlapping signaling pathways that regulate specific metabolic pathways, such as in gene knockouts of peroxisome proliferator-activated receptor (PPAR)-α versus -β/δ versus -γ signaling, but also subtle differences in transcriptional targets.[326,334,341–351] Moreover, overexpression studies have highlighted important differences in pathways that can be induced when these transcriptional regulators are overexpressed. Genetic models have also provided interesting insights between mutations that affect nonmetabolic signaling pathways, such as those that regulate excitation-contraction coupling or sarcomeric function and those that govern metabolism.[118,327] Lastly, recent studies have exposed important cross talk between the expression of genes within the cardiomyocytes and those that regulate metabolism in peripheral organs, such as adipose tissue or skeletal muscle.[4,5] The ultimate goal of genetic modeling remains, however, to recapitulate human disease. In this regard, the modeling of the mitochondrial metabolic disorder Barth syndrome using iPSCs and "heart on chip" technologies is noteworthy.[352]

Nongenetic Models for Cardiac Metabolism

Nongenetic models assess the consequences of metabolic, neurohumoral, hemodynamic, and ischemic stresses on cardiac metabolism and energy transfer and efficiency. Although it is not possible to be complete, a comprehensive list is given in the Appendix (Table A5).

Research performed throughout the history of intermediary metabolism has revealed that qualitative and quantitative changes affect the metabolic homeostasis of the mammalian body.[353] These changes may or may not result in changes of cardiac structure and function, whereas dietary interventions can have dramatic effects on cardiac energy metabolism. Although there is still a perceived gap between cardiac metabolism and cardiovascular health, there is considerable clinical interest in the components of cardioprotective diets.[2] Critical issues include the specific composition of the individual diets, the duration of feeding of a specific diet or the duration of caloric restriction, and the end points to be assessed. The first part of this section summarizes the salient features of specific diets on the heart. The second part of this section highlights salient changes of cardiac metabolism in response to selected pharmacological agents.

Dietary Undernutrition

Fasting and Starvation

The terms *fasting* and *starvation* are often used interchangeably, but they are not the same. In general, fasting is considered a short-term abstinence from caloric food intake, whereas starvation is a long-term, severe deficiency in caloric food intake. Biochemically, a distinction between fasting and starvation is defined by the inflection point at which glycogen stores are exhausted, muscle protein degradation is accelerated, and/or ketone body production increases markedly. The time from fasting to initiation of starvation differs between species (eg, a 12-hour food withdrawal in rats increases plasma ketone bodies 5-fold but has little effect in humans). Both fasting and starvation result in a (paradoxical) increase in cardiac glycogen stores and an increased reliance on FAs as fuel for

respiration as adipocyte lipolysis is stimulated, thus increasing nonesterified FAs. Fasting and starvation also increase the transcription of FA oxidative enzymes within multiple tissues, including the heart.[354] Thus, the contribution of FA oxidation as a source of energy for the heart increases. During starvation, liver ketogenesis increases the availability of this fuel as a source of myocardial ATP production. Starvation also results in increased protein degradation, decreased protein synthesis, reduction in contractile protein subunits, and cardiac atrophy.[355] To date, no uniformity exists on protocols for studies regarding the impact of fasting or starvation on cardiac metabolism. Parameters to consider (and reported in the literature), besides the duration of food withdrawal or restriction, include the time of day at which fasting is initiated and the housing conditions (eg, bedding versus wire-bottom cage). It is of note that fasting paradoxically raises myocardial glycogen content,[356,357] and metabolic switches induced by fasting or starvation may affect cardiac function in response to pathological stresses such as ischemic injury,[358] although the literature here is contradictory.[357,359]

Caloric Restriction

Caloric restriction has received attention as a means of improving cardiometabolic health (including adiposity, glucose homeostasis, insulin sensitivity, and cardiac function/tolerance to adverse stresses) and longevity. In animal models, the degree of caloric restriction routinely performed is in the range of 10% to 50%, although the duration of caloric restriction may vary markedly from an overnight fast to the lifespan of the organism. Strategies for caloric restriction may also vary in different experimental settings; common methods include providing a meal of reduced caloric content on a daily basis, whereas others alternate between a day of feeding and a day of fasting. In the rodent, both protocols involve cycling between feeding and fasting as a function of food availability. In terms of cardiac metabolism, the contribution of FA oxidation to ATP production does increase after caloric restriction; however, the heart remains very sensitive to insulin, and carbohydrate oxidation rates are high during periods of meal consumption. Caloric restriction has many desirable effects directly on the heart, including decreased hypertrophic signaling, decreased adverse remodeling after myocardial infarction, and increased ischemia tolerance. This may be related to the caloric restriction–induced increase in insulin sensitivity, decreased TG turnover, increased cardiac AMPK activity, decreased mitochondrial acetylation, decreased myocardial oxidative stress, or increased autophagy.[360–366]

Vitamin and Mineral Deficiencies

Human and animal studies on vitamin and mineral deficiencies implicate changes in cardiac energy metabolism and function. For instance, vitamin B_1 deficiency in humans increases blood lactate and pyruvate levels, a finding consistent with an increase in glycolysis and a decrease in glucose oxidation.[367] In addition, many vitamin deficiencies observed in humans can be mimicked in animal models through appropriate dietary manipulations, and the impact on cardiac metabolism can be assessed by standard techniques. For example, vitamin D deficiency results in increased glycolysis and decreased mitochondrial oxidative metabolism in the rat heart. The perturbations in cardiac metabolism are accompanied by increased oxidative stress, apoptosis, inflammation, and cardiac hypertrophy, as well as decreased ejection fraction.[368] Animal models of mineral deficiency such as copper deficiency[369] or selenium deficiency[370] are also associated with increased hypertrophy, decreased cardiac function, increased oxidative stress, and increased endoplasmic reticulum stress. However, the relationship between alterations in contractile function and cardiac energy metabolism in these studies has not been clearly delineated.[371]

Dietary Overnutrition

High-Fat Diets

Feeding rodents a high-fat diet is a very common experimental approach to inducing obesity and impaired glucose homeostasis. For instance, feeding mice a diet high in fat (45% or 60% calories from FAs) for 8 to 10 weeks results in obesity, glucose intolerance, and whole-body insulin resistance. Additionally, alterations in cardiac metabolism are observed, including cardiac insulin resistance, an increase in cardiac FA oxidation, and a concomitant decrease in glucose oxidation.[372] These metabolic changes precede the onset of cardiac dysfunction, which typically occurs 15 to 20 weeks after the onset of a high-fat diet. It is not clear whether the metabolic changes that occur early in this model of dietary overnutriton contribute to the eventual contractile dysfunction that occurs in these hearts. Some reports suggest that a high-fat diet can actually protect the heart from ventricular expansion and contractile dysfunction if mice are subjected to heart failure attributable to pressure overload, myocardial infarction, or genetic cardiomyopathies.[373,374] This protection has been attributed to an upregulation of FA oxidation and a preservation of mitochondrial oxidative metabolism.

The cardiovascular effects of diets high in polyunsaturated FAs remain to be fully delineated. Studies have suggested that feeding rodents a diet high in polyunsaturated FAs (a mixture making up 39% of total FAs) increases lipid peroxidation and lipid peroxide accumulation, as well as decreasing Krebs cycle activity.[375] However, many other studies have shown that diets high in n-3 polyunsaturated FAs (docosahexaenoic acid, eicosahexaenoic acid) decrease the severity and progression of heart failure.[374] This is associated with reduced levels of circulating TGs, decreased inflammation, reduced levels of cytokines, decreased cardiac mitochondrial permeability transition pore opening, and improved mitochondrial function.[376] What effect diets high in polyunsaturated FAs have on metabolic flux in the heart has not been investigated extensively, but the conceptual framework of a nutritional treatment for heart failure is both timely and attractive.

High-Sugar Diets

The cardiovascular effects of high-sugar diet have also been studied, yielding an array of results. High-fructose diets will cause obesity and cardiac insulin resistance,[377,378] as well as alterations in myocardial calcium handling.[379] Although a high-fructose diet has been shown to exacerbate heart failure in mice when the heart is subjected to pressure overload,[380] a similar diet in genetic models of cardiomyopathy does not exacerbate heart failure.[381] This lack of detrimental effect of a

high-sugar diet occurs despite the presence of increased serum lipids and myocardial superoxides. Contrary to expectations, a high-sucrose diet (68% of total caloric intake) improves cardiac efficiency in rat hearts.[382] Although this feeding protocol increases cardiac insulin resistance, it improves the coupling of glycolysis to glucose oxidation, thereby enhancing cardiac efficiency. In contrast, ischemic tolerance is reduced in insulin-resistant hearts from sucrose-fed animals.[383]

High-Protein Diets

High-protein diets containing 30% of energy intake in the form of protein (as opposed to 18% protein in standard diet) have no effect on cardiac stress and function in rats 22 weeks after aortic constriction and significantly decrease survival.[384] The deleterious effects of high-protein/low-carbohydrate diets on the cardiovascular system appear to increase with age.[385] On the other hand, experience with the effects of a low-protein diet (0% of energy intake) on the heart is still limited only to branched-chain amino acid metabolism.[386] In short, more research is needed to delineate the effect of specific diets on both the short- and long-term function of the heart.

Pharmacological Agents

The list of pharmacological agents that affect cardiac metabolism is both long and varied. In the Appendix, we limit the review to newer models of streptozotocin-induced diabetes mellitus, methylglyoxal, and other representative drugs that affect cardiac metabolism.

Metabolism of the Developing, Newborn, and Neonatal Heart

General Technical Considerations for Performing In Situ Experiments in Newborn Animals

The study of metabolic activities in fetal and newborn hearts presents special challenges. Because of the level of maturation and their smaller size, thermoregulation is not as efficient as in adult animals. Anesthetized newborn or immature sheep and pigs show a steady decline in temperature, particularly during open-chest experiments. Thus, it is mandatory that normothermic temperature is maintained by either warming blankets or heating pads to prevent systemic cooling and decreases in myocardial oxygen consumption. Please refer to the Appendix for further details and a brief historical synopsis.

^{31}P NMR has played an important role in defining developmental changes in energy-rich phosphate kinetics and regulation of mitochondrial respiration in situ. However, phosphates in blood contribute to the myocardial ^{31}P signal and contaminate the inorganic phosphate peak. This is a particular issue in newborn sheep with high red blood cell 2,3-diphosphoglycerate content. The 2,3-diphosphoglycerate can be separated out with high-frequency NMR (phosphorous at 81 MHz, 4.7 T), and this contamination resolves by 4 weeks of age.[387] Delivery of stable isotopes to the immature heart in situ for either ^{13}C NMR or LCMS-GCMS studies presents several challenges. Although the NMR method offers the advantage of using multiple labeled substrates to determine the relative fractional contribution from each, the more specific GCMS methods are generally limited to evaluating the contribution of a single labeled substrate at a time. Unlike in isolated perfused heart studies, complete (or near 100%) isotope enrichment in myocardium cannot be achieved in situ.[388] However, reasonable isotope enrichment must be attained to provide adequate signal to noise and data quality. In large adult animals, relatively high isotope enrichment can be attained by using selective coronary perfusion through an extracorporeal shunt from the femoral artery, where flow and isotope concentration can be closely controlled.[389] Because of the limiting size of the small arterial vessels in newborns or neonatal animals, this is technically not feasible. Accordingly, high isotope enrichment in the heart can only be accomplished by direct infusion via a catheter in a coronary artery. Generally, this is performed by catheter insertion through a branch of the left anterior descending coronary artery in neonatal piglets. Reasonable isotope enrichment often requires infusion with supraphysiological concentrations of substrate, which is of some concern.[388] However, relative contributions of substrates (long-chain free FAs [FFAs], medium-chain FFAs, and lactate) do not differ from those achieved by systemic isotope infusion at physiological concentrations with much lower tissue enrichment.[123] Alternatively, $^{13}CO_2$ production in the coronary sinus and measurement by GCMS may be performed after systemic infusion of ^{13}C-labeled substrate. This methodology also requires single substrate infusion per animal, as well as complex procedures for collection of CO_2.[390] Comparisons of immature rodents and large animals are shown in the Appendix.

Metabolic Studies in Isolated Newborn Heart

As already mentioned, the small size of the newborn heart in rodents makes isolated working heart perfusions technically challenging. For this reason, investigators have turned to isolated perfused heart studies in newborns of larger species, such as the rabbit[391] or pig.[392] However, there are inherent problems using the isolated working heart in the newborn. In particular, there are anatomic and functional differences between the newborn and adult heart, which include a larger proportional size of the right ventricle in relation to the left ventricle than in the adult heart. The classic isolated working heart preparation involves cannulation of the left atrium and filling of the left ventricle, without filling of the right ventricle. To overcome this problem, Werner and colleagues developed an isolated biventricular working heart for newborn pigs,[393] whereas the Lopaschuk group developed an isolated biventricular working rabbit heart preparation.[391,394] Using these models, benchmark experiments using ^{14}C-isotope labeling revealed a rapid increase in FA uptake and oxidation by the heart that occurred shortly after birth. Glucose contribution to ATP production decreased from almost 60% to 10% in rabbit heart between postnatal days 1 and 7.[391] The increase in FA oxidation in the newborn rabbit heart correlated directly with a reduction in cardiac levels of malonyl-CoA, an inhibitor of CPT-1, secondary to an increase in malonyl-CoA decarboxylase activity. This ability to increase FA oxidation after birth in the rabbit heart is impaired during pressure- and volume-overload–induced hypertrophy, which results in the heart remaining in a more "fetal" metabolic state.[395]

The Newborn Heart: Clinical Translation

Clinical problems in the newborn and neonate that are related to metabolism vary substantially from the issues prevalent in adults. In particular, myocardial protection and support after neonatal cardiac surgery remains an important area for improvement and a focus for investigators. For instance, complex congenital heart defect surgery often requires long aortic cross-clamp periods, full circulatory arrest, or both. Recent studies in a neonatal pig model have highlighted the role of metabolic flexibility in maintaining cardiac function after stress conditions such as ischemia-reperfusion associated with such surgeries[117] or during weaning from extracorporeal membrane oxygenation.[124,396] Specifically, promotion of substrate oxidation via pyruvate dehydrogenase improves neonatal cardiac function under these conditions, which emulate clinical scenarios.[117,123,124,396–399] Thyroid hormone supplementation elevates flux through this oxidative pathway and has produced clinical improvement in a major clinical trial in infants undergoing cardiopulmonary bypass.[400]

Ischemia, Reperfusion, Heart Failure, and Metabolic Modulation

Alterations in cardiac energy metabolism contribute to the severity and complications associated with many forms of heart disease, including ischemic heart disease and heart failure.[401] Metabolic modulation of cardiac energy metabolism aims to lessen the severity of heart disease. This includes increasing overall energy supply to the heart, as well as optimizing the use of a particular energy substrate by the heart. The use of pharmacological agents to modify cardiac energy metabolism also provides important insights into the close relationship between FA and carbohydrate metabolism in the heart. For instance, pharmacological inhibition of FA oxidation results in a parallel increase in glucose oxidation in the heart and may provide a means to increase the efficiency of ATP production[402] and thus improve function in the ischemic heart.

The approach of metabolic modulation as a strategy to treat heart disease has been examined in many experimental models. One of the earliest examples of metabolic modulation to be translated to humans involved the use of glucose-insulin-potassium therapy to treat ischemic heart disease. The proposed effects of glucose-insulin-potassium therapy were thought to occur via increasing rates of glycolysis, as well as decreasing circulating concentrations of FFAs.[403,404] However, clinical results from human studies were equivocal, with some studies showing clinical benefit, whereas others did not.[405] As a result, there is not a clear consensus as to whether glucose-insulin-potassium therapy is beneficial in the treatment of acute myocardial infarction.

A number of pharmacological agents can impact cardiac energy metabolism by altering FFA supply to the heart. This includes the use of agonists of the PPAR ligand–activated nuclear hormone receptors (PPARα, PPARγ, and PPARβ/δ). Fibrates, which are PPARα agonists, decrease circulating FFAs, mostly by increasing hepatic expression of FA oxidation enzymes.[406] In contrast, PPARγ ligands promote lipid sequestration in adipose tissue. The consequent decrease in circulating FFA concentration and decreased FFA extraction in the heart can decrease myocardial FA β-oxidation, concomitant with increased glucose uptake and oxidation.[407,408] Despite the favorable effects observed in experimental settings, clinical trials investigating the use of PPAR agonists have raised concerns about the safety of their use. Niacin and nicotinic acid can also be used to decrease circulating FA levels and have been shown to have beneficial effects in heart disease.[409] However, side effects such as effects on blood pressure have limited the clinical application of this approach.

Potential strategies for metabolic modulation also involve direct alterations in enzymes involved in controlling energy metabolism in the heart. One such enzyme is carnitine palmitoyltransferase-1 (CPT-1), which is a key enzyme involved in mitochondrial FA uptake. Inhibition of CPT-1 has been used as an approach to inhibiting myocardial FA oxidation. CPT-1, in turn, is inhibited by malonyl-CoA, and experimental strategies to increase malonyl-CoA levels (by inhibiting malonyl-CoA decarboxylase, which is involved in malonyl-CoA degradation) protect the ischemic heart and the failing heart.[401] Clinical studies on the effect of malonyl-CoA decarboxylase inhibitors in heart disease, however, have not yet been conducted. Direct inhibition of CPT-1 with etomoxir, oxfenicine, and perhexiline also shows protective effects in the ischemic and failing heart. Limited clinical studies with etomoxir and perhexiline have suggested potential use in ischemic heart disease and heart failure.[410–412] However, a clinical trial with etomoxir was prematurely terminated because of hepatic toxicity of the drug. Although perhexiline enjoys limited use as an antianginal agent, further clinical studies are required to determine its potential clinical efficacy in treating heart failure.

Another approach to inhibit mitochondrial FA oxidation is to directly inhibit enzymes of FA β-oxidation. An agent that does this is trimetazidine, a partial FA β-oxidation inhibitor that competitively inhibits long-chain 3-ketoacyl CoA thiolase.[413] Trimetazidine inhibition of FA oxidation is accompanied by an increase in myocardial glucose oxidation. A meta-analysis of clinical studies in angina pectoris have confirmed the effectiveness of trimetazidine as an anti-ischemic agent.[414] A meta-analysis of trimetazidine has also suggested trimetazidine may be an effective strategy in treating heart failure.[415]

Directly increasing myocardial glucose oxidation may be another approach to improve cardiac function.[416] Dichloroacetate (DCA) acts by direct stimulation of mitochondrial pyruvate dehydrogenase complex via inhibition of the activity of pyruvate dehydrogenase kinase. The improved coupling between glycolysis and glucose oxidation is believed to be the mechanism by which DCA exerts its cardioprotective effects.[401] Experimental studies show that DCA has beneficial effects in ischemic heart disease and heart failure, but clinical data are scarce. Acute DCA administration to patients with coronary artery disease does improve left ventricular stroke volume in the absence of changes in heart rate, left ventricular end-diastolic pressure, or myocardial oxygen consumption.[417] However, chronic use of DCA to treat heart disease is complicated by potency and pharmacokinetic issues associated with DCA. A number of pyruvate dehydrogenase kinase inhibitors are presently being developed.

Metabolism and CVD: Epidemiological, Genomic, Plasma Proteomic, and Metabolomic Approaches

Dysregulated systemic metabolism affects every organ of the body, including the heart. A synopsis of discovery strategies relevant to cardiac disease risk and metabolism is therefore in order. It places metabolism in a wider context. At the same time, the metabolic information becomes clinically relevant.

It is widely accepted that independent prospective associations have identified core risk factors for CVD, such as hypertension and hyperlipidemia. This identification has resulted in their use for prognostication, with the development of prediction equations combining such factors to stratify future risk of CVD; these have served as the cornerstone of clinical guidelines for use of preventive therapies. However, because a substantial proportion of people who develop CVD have no identifiable risk factors, improved CVD prediction rests with discovery of new disease pathways and biomarkers that are "orthogonal" to existing ones. The vast array of data available through genomics, transcriptomics, proteomics, and metabolomics offers new opportunities for discovery and will require new bioinformatic and analytic approaches to chart the intricate networks responsible for the pathogenesis of complex diseases such as CVD. One approach is genome-wide association and next-generation sequencing studies. Another approach is the profiling of plasma metabolites. A discussion of genomics and metabolomics, and cardiovascular risk assessment is presented in the Appendix.

Metabolomics and Cardiovascular Risk Assessment

Plasma metabolites, proteins, and other molecules provide a valuable window on the endogenous and exogenous factors that influence cardiometabolic risk, adding information that is complementary to knowledge of fixed genetic determinants. Platforms that enable the global profiling of proteins (proteomics) or small molecules (metabolomics) hold particular promise for clinical biomarker discovery. Details have been described previously. These technologies permit simultaneous interrogation of multiple pathways in a relatively unbiased manner (not limited to pathways already known to contribute to CVD), which increases the probability of finding orthogonal information to aid risk assessment and reveal new mechanisms.[418] The Appendix contains a full discussion of this emerging field.

Metabolic Signaling in Heart Disease: The Enduring Need for Quantitation

The biochemistry of energy substrate metabolism is a dynamic and complex facet of cardiac biology. The logic of cardiac metabolism has moved from the concept of a dynamic "power grid" to a far more comprehensive model that includes metabolic signals as mediators of functional adaptation and maladaptation of the heart. A fitting example is acetyl-CoA, a prototype link between metabolism and epigenetic modifications.[419] As analytical tools become more diverse, their strategic deployment becomes imperative in the search for novel cellular mechanisms underpinning the function of the heart in the pursuit of novel targets for drugs, and even for cardiovascular risk stratification. In a brief sketch on the evolution of metabolism, the late Larry Holmes wrote, "Criteria were set out and guided scientists through years of detailed investigation of a special part of nature before these investigations attained any success dramatic enough to qualify as a paradigm. The paradigm achievements grew out of the general acceptance of these methods, criteria, and assumptions, rather than the other way around."[420] In other words, in a complex, dynamic system such as cardiac metabolism, it is not possible to expose a mechanism without qualitative and quantitative assessment of all biological processes. In spite of the complexities of intermediary metabolism, this principle is not likely to change as new discoveries are made. In metabolic methodology, as in metabolism itself, all is in flux. Yet, the need for rigor and reproducibility remains a permanent challenge.[421]

Acknowledgments

The authors wish to recognize Drs Giovanni Davogustto, Anja Karlstaedt, and Joost J Luiken for contributing to some of the sections, Roxy A. Tate for help during the early stages, and Truong N. Lam for help during the final stages of the work.

Disclosures

Writing Group Disclosures

Writing Group Member	Employment	Research Grant	Other Research Support	Speakers' Bureau/ Honoraria	Expert Witness	Ownership Interest	Consultant/ Advisory Board	Other
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This table represents the relationships of writing group members that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all members of the writing group are required to complete and submit. A relationship is considered to be "significant" if (a) the person receives $10 000 or more during any 12-month period, or 5% or more of the person's gross income; or (b) the person owns 5% or more of the voting stock or share of the entity, or owns $10 000 or more of the fair market value of the entity. A relationship is considered to be "modest" if it is less than "significant" under the preceding definition.

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Reviewer Disclosures

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Joanne Ingwall	Harvard Medical School	None	None	None	None	None	None	None
Stephanie Nakano	University of Colorado School of Medicine, Children's Hospital Colorado	None	None	None	None	None	None	None
Naveed Sattar	University of Glasgow (United Kingdom)	None	None	None	None	None	Amgen*; Sanofi*; AstraZeneca*; Eli Lilly*; Boehringer Ingelheim*	None

This table represents the relationships of reviewers that may be perceived as actual or reasonably perceived conflicts of interest as reported on the Disclosure Questionnaire, which all reviewers are required to complete and submit. A relationship is considered to be "significant" if (a) the person receives $10 000 or more during any 12-month period, or 5% or more of the person's gross income; or (b) the person owns 5% or more of the voting stock or share of the entity, or owns $10 000 or more of the fair market value of the entity. A relationship is considered to be "modest" if it is less than "significant" under the preceding definition.

*Modest.

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Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Assessing Cardiac Metabolism: A Scientific Statement From the American Heart Association

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on behalf of the American Heart Association Council on Basic Cardiovascular Sciences

Circ Res. 2016;118:1659-1701; originally published online March 24, 2016;
doi: 10.1161/RES.0000000000000097
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Correction

In the article by Taegtmeyer et al, "Assessing Cardiac Metabolism: A Scientific Statement From the American Heart Association," which published ahead of print March 24, 2016, and appeared in the May 13, 2016, issue of the journal (*Circ Res.* 2016;118:1659–1701. doi: 10.1161/RES.0000000000000097), a correction was needed.

On page 1689, in the Writing Group Disclosures table, the fourth footnote read, "§Dr Recchia is now at Lewis Katz School of Medicine, Temple University Scuola Superiore Sant'Anna, Pisa." It should have read, "§Dr Recchia is now at the Lewis Katz School of Medicine, Temple University, Philadelphia, and at the Scuola Superiore Sant'Anna, Pisa, Italy."

This correction has been made to the print version and to the online version of the article, which is available at http://circres.ahajournals.org/content/118/10/1659.full.

(*Circ Res*. 2016;118:e35. DOI: 10.1161/RES.0000000000000106.)

Appendix to AHA Scientific Statement

Assessing Cardiac Metabolism

APPENDIX 1. Historical Notes on Metabolic Networks

Cardiac metabolism is a book with many chapters. Very readable, general, introductions to the evolution of energy substrate metabolism in biological systems have appeared in the 1980s and 1990s.[1, 2] Reviews on cardiac energy substrate metabolism provide further detail to the contemporary understanding of energy transfer in the heart.[3-12] Here, and elsewhere, the reader will appreciate that the intermediary metabolism of energy providing substrates is complex and has been under investigation for more than a century. Renewed interest in cardiac metabolism is based on the recognition that the intermediary metabolism of energy providing substrates forms an integral part of cellular adaptation and maladaptation to changes in the heart's environment. Alternatively stated, metabolism offers an integral approach to understanding how the heart functions.

APPENDIX 2. Expanded Notes on Metabolic Pathways and Networks in the Heart

a) Pathways of Fatty Acid Metabolism in the Heart

The first major fuel source of the heart are fatty acids, which originate from lipolysis of triacylglycerol (TG) contained in circulating chylomicrons and very low density lipoprotein (VLDL), from free fatty acids circulating in the blood, or from endogenous TG present in the form of intracellular lipid droplets.[11] The myocardial uptake of fatty acids from the circulation occurs either by passive diffusion or by a facilitated transport process involving membrane proteins such as FAT/CD36, the fatty acid transport protein (FATP), and the cytoplasmic fatty

acid binding protein (FABP). By controlling fatty acid uptake these membrane proteins are pivotal in regulating the rate of myocardial fatty acid utilization.[13] In the cytosolic compartment, fatty acids are activated via esterification to CoA, thus generating a fatty acyl-CoA moiety that can be channeled into distinct metabolic fates including phospholipid and TG synthesis, signal transduction, and mitochondrial fatty acid ß-oxidation. Peroxisomal fatty acid oxidation, although present in the heart, is a minor contributor to ATP production.

A key regulatory step in the metabolic network that modulates the contribution of fatty acids as a cardiac energy source is the transfer of fatty acyl groups across the inner mitochondrial membrane. Carnitine palmitoyl transferase 1 (CPT1) is located on the outer mitochondrial membrane and converts fatty acyl-CoA molecules to their respective fatty acyl-L-carnitine moieties. The latter are shuttled into the mitochondrial matrix, followed by re-conversion to fatty acyl-CoA, which subsequently undergoes ß-oxidation. CPT1 is potently inhibited by malonyl-CoA, providing a key control site for fatty acid ß-oxidation. Malonyl-CoA levels are, in turn, regulated by cardiac workload and neuro-hormonal factors through fluctuations in its rates of synthesis and degradation.

In addition to transporters, the activities of the enzymes of mitochondrial fatty acid ß-oxidation also influence the overall rates of fatty acid ß-oxidation. The acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase enzymes are both sensitive to ratios of $FAD/FADH_2$ and NAD^+/NADH in the mitochondrial matrix, while the enzyme 3-ketoacyl-CoA thiolase is sensitive to the mitochondrial acetyl-CoA/CoA ratio. As a result, alterations in these intermediates either due to perturbations in Krebs cycle activity, ETC activity, or oxidation of competing substrates can alter the rates at which fatty acid ß-oxidation supplies acetyl-CoA to the Krebs cycle and reducing equivalents to the ETC.

b) Pathways of Carbohydrate Metabolism in the Heart

The second major group of fuels used by the heart are carbohydrates. Predominant substrates in this group are glucose and lactate. Glucose use is initiated via its cellular uptake, which is a complex process coupled to the rates of glucose delivery to the heart, glucose transport into cardiomyocytes, and glucose phosphorylation in the cytosolic compartment. In the heart, glucose uptake occurs via facilitative transport, mediated by the family of glucose transporters (GLUTs), with GLUT1 and GLUT 4 dominant in cardiac muscle. Once inside the cell, hexokinase (predominantly its isoform II) phosphorylates glucose, generating glucose 6-phosphate (G6P). G6P then has a number of potential fates, which include: 1) glycolysis, 2) glycogen synthesis, 3) the hexosamine biosynthetic pathway (via fructose 6-phosphate), or 4) the pentose phosphate pathway which generates NADPH and ribose 5-phosphate. This latter pathway is important in supporting anabolic activities in the heart (especially in the developing or the stressed heart) and in the regulation of reactive oxygen species formation via NADPH oxidases.

Glucose delivery and glucose uptake are the first steps in the glycolytic pathway. Inside the cardiomyocyte, the glycolytic pathway is highly regulated by ATP/AMP ratio, H^+ levels, P*i* levels, citrate levels, the redox state, allosteric regulators such as G6P, ATP, citrate or fructose 2,6-bisphosphate, and also by transcriptional control, and post-translational regulation. When cardiac energy demand increases, and/or mitochondrial ATP production decreases,rates of glycogenolysis, glucose uptake, and glycolysis increase.[14, 15] The acute increase in workload of the heart or the metabolic state of exercise result in the preferential oxidation of carbohydrate fuels accelerate.[16, 17]

The fate of glycolysis-derived pyruvate represents further crossroads in the cardiac metabolic network. Under aerobic conditions, pyruvate is transported into the mitochondria via a mitochondrial pyruvate carrier, where it either undergoes oxidative decarboxylation by the pyruvate dehydrogenase (PDH) complex (yielding acetyl-CoA). Alternatively, pyruvate is carboxylated to oxaloacetate by pyruvate carboxylase and/or to malate by malic enzyme (thereby acting as an anaplerotic substrate for the Krebs cycle). The PDH complex (PDC) has a critical function in providing acetyl-CoA for the Krebs cycle as well as reducing equivalents for the ETC. PDH is inhibited by its products as well as by phosphorylation by PDH kinases. In the cardiac metabolic network, this control of the PDC is an important determinant of how competing substrates influence carbohydrate oxidation. For instance, an increase in [NADH]/[NAD^+] and/or in [acetyl-CoA]/[CoA] will decrease PDC activity, but will increase pyruvate carboxylation. Carboxylation of pyruvate via malic enzyme and/or pyruvate carboxylase is concomitant with the oxidative decarboxylation of pyruvate and results in the generation of the Krebs cycle intermediates malate and oxaloacetate, referred to as anaplerosis.[18, 19] In contrast, a decrease in the [NADH]/[NAD^+] and/or [acetyl-CoA]/[CoA] ratios (which decreases PDH kinase), or an increase in PDH phosphatase activity, dephosphorylates and activates PDH, thereby stimulating PDH flux. In addition to allosteric and covalent control, PDH and PDH kinase are also under a high degree of transcriptional and post-translational control. It is also important to note that in response to increased circulating levels lactate acts as an allosteric inhibitor of the glycolytic pathway at the level of the enzyme glyceraldehyde 3-phosphate dehydrogenase and becomes the major fuel for respiration of the heart in the state of exercise.[20]

c) Ketone Body Metabolism in the Heart

During periods of starvation or with consumption of low carbohydrate/high fat diets, ketone bodies become an important energy source for the heart. A key determinant of their rates of oxidation by the heart is the concentration of acetoacetate and β-hydroxybutyrate in the blood.[3] The reason seems straightforward: ketone bodies readily enter the heart and are readily transferred to the mitochondria. β-hydroxybutyrate is oxidized to acetoacetate by β-hydroxybutyrate dehydrogenase 1 (BDH1). Acetoacetate is activated to acetoacetyl-CoA by succinyl-CoA:3-oxoacid-CoA transferase (SCOT). This acetoacetyl-CoA is then subjected to a thiolysis reaction to provide acetyl-CoA for the Krebs cycle. Within the cardiac metabolic network, increased ketone body oxidation during ketotic states is very effective at inhibiting both carbohydrate and fatty acid ß-oxidation, primarily as a result of an increase in $NADH/NAD^+$ and/or acetyl-CoA/CoA production. Transcriptional regulation of enzymes involved in ketone oxidation (BDH1 and SCOT) as well as post-translational modification (acetylation and tyrosine nitration) also are important in regulating the oxidation of ketone bodies.[21]

d) Amino Acid Metabolism in the Heart

Additional sources of acetyl-CoA for the Krebs cycle are amino acids, particularly the branched chain amino acids (BCAAs), leucine, isoleucine, and valine. After their transamination, degradation of the β-keto acid carbon skeleton occurs via the branched chain-keto acid dehydrogenase complex. The complex converts the β-keto acids into acyl-CoA derivatives, which undergo ß-oxidation to produce either acetyl-CoA or succinyl-CoA (another anaplerotic substrate) that enter the Krebs cycle. Although not a major source of ATP production in the heart, when plasma levels are low, BCAA oxidation increases amino acid concentrations increase (e.g., after a high protein meal, or during starvation). BCAA also impact insulin

signaling in the heart, which, in turn, impacts fatty acid, carbohydrate, and amino acid utilization. Lastly, defects in BCAA metabolism are a feature of metabolic remodeling in the failing heart.[22]

APPENDIX 3. Expanded Notes on Systems Biology and Mathematical Modeling of Cardiac Metabolism

a) Stoichiometry-based Models

Metabolic flux analysis (MFA)

The metabolic response of cardiomyocytes to varying external conditions (heart rate, work load, nutrient and hormone concentrations) is accomplished by orchestrated changes in the fluxes through pathways of the underlying biochemical reactions. With the exception of directly measurable uptake and release rates, quantification of internal metabolic fluxes requires the administration of (preferentially ^{13}C-) labeled tracer metabolites and determination of the isotope enrichment in those metabolites, which become labeled during the passage of the tracer through the network.[23] Determination of ^{13}C-isotopomer distributions by NMR spectroscopy allows for the non-invasive quantification of metabolic fluxes in central metabolic pathways, including glycogen synthesis, glycolysis, and the citric acid cycle. Converting measurements of isotope enrichments into reliable estimates of flux rates has to be based on network models encompassing all relevant routes of label distribution. Usually, there are numerous flux feasible distributions, which may describe the data so that extensive Monte Carlo sampling (computational algorithms which rely on repeated random sampling to obtain numerical results) has to be performed to determine confidence regions for the estimated fluxes.[24] Whereas MFA-based investigations covering several pathways of intermediary metabolism are quite common for relatively simple cellular systems like *E. coli* and yeast there are still only few such applications to cardiomyocytes. [25-28]

b) Constraint-based modeling (CBM)

CBM (also often termed flux-balance analysis, FBA) is a method to predict stationary metabolic fluxes from optimization principles.[29] The method rests on the assumption that the regulation of metabolic systems has been shaped through natural evolution to meet central functional requirements, e.g. the growth of a microorganism, or the transfer of energy in the cardiomyocyte in an optimal manner. This optimality assumption is formulated by introducing an objective function relating the metabolic fluxes to a physiological function and to determine the fluxes such that this objective function adopts an optimal value. CBM is only applicable under the constraint of stationary metabolic states. Further constraints on fluxes can be set by the maximal capacities of enzymes and transporters. Useful applications of CBM include: i) Prediction of flux changes associated with gene expression changes. For example, constraining a whole-cell reconstruction of the metabolic network of *Drosophila's* heart by gene-expression data obtained under normoxic and hypoxic conditions revealed that hypoxia-adapted flies may divert pyruvate flux better through pyruvate dehydrogenase than through pyruvate carboxylase (http://escholarship.org/uc/item/7x7764kk); ii) Assessment of the maximal yield of a specific target metabolite (e.g. ATP or the membrane lipid cardiolipin) achievable with different mixtures of alternative substrates. Such analyses may be particularly useful for the purposeful supplementation of yield-limiting nutrients.[30] The striking advantage of CBM is its simplicity: It requires only knowledge of the stoichiometry of the network and thus can be applied to whole-cell network reconstructions. However, the predicted flux distributions are hypothetical owing to the subjective choice of the flux objectives and the entire neglect of the kinetic and regulatory properties of enzymes and transporters.

c) Kinetic Models

Kinetic modeling is the unrivalled computational method to explain the function of metabolic systems by the properties and interactions of the underlying biochemical and biophysical processes. Kinetic modeling of heart metabolism has a long history beginning in the late 1970s with the pioneering work of Garfinkel and coworkers.[31, 32] These models were based on simple enzymatic rate laws of the Michaelis-Menten type and comprised the pathways glycolysis, citric acid cycle and beta oxidation. Cortassa [33] developed an integrative model of cardiac mitochondrial metabolism, including the citric acid cycle, oxidative phosphorylation and calcium concentrations. Further extensions of this model[34-36] coupled the energy metabolism to mitochondrial electrophysiology and external blood flow. However, currently available kinetic models lack many aspects of cardiac metabolism that are particularly relevant to the failing heart, including the varying utilization of alternative energy-delivering substrates (glucose, lactate, fatty acids, ketone bodies), the hormonal regulation of cardiac metabolism by insulin, epinephrine, norepinephrine, the accumulation of TG and ceramide in obesity as potential sources of lipotoxicity, the pathways involved in the elimination of reactive oxygen species (e.g. pentose phosphate pathway), and the salvage metabolism of purine being essential for the rescue of energy-rich phosphates in ischemia.

APPENDIX 4. Expanded Notes on Measuring Metabolism

The heart is a metabolic omnivore, and complex interactions occur within the entire cardiometabolic network that determines the relative amount of fatty acids, glucose, lactate, ketone bodies, or amino acids that are used as a fuel source for ATP production. As discussed, substrate supply to the heart and rates of uptake are the determinants for the relative amount at

which each of the carbon substrate is used by the heart. In addition to the extracellular sources of fatty acid and glucose, the heart also contains sizeable stores of fatty acids in the form of TGs, as well as intracellular stores of glucose contained in the form of glycogen. The fatty acids and glucose within these intracellular stores are subjected to turnover, and especially glycogen can be rapidly mobilized in times of need (e.g., in times of increased energy demand or during hormonal stimulation). See **Table A1** of the **Appendix** for more information on metabolite concentrations in the rat heart.

Metabolic research has witnessed an unprecedented rate of growth in the age of 'omics', during pursuit of genomic, transcriptomic, proteomic and metabolomics data interpretation. These comprehensive data sets have confirmed the simple concept originally founded on painstaking biochemical research during the last century: Metabolic pathways are a series of enzyme catalyzed reactions that convert a substrate into a product. All, or almost all, of the constituent reactions of pathways have been fully elucidated in the "classical era" of biochemistry in a variety of models, mostly tissue homogenates or thin tissue slices in the Warburg Manometer. Experimental strategies included the addition of possible intermediates of a pathway, the identification of intermediates which accumulate or disappear upon addition of selective inhibitors of distinct points within the pathway. This research was subsequently confirmed in whole organs using the addition of a labeled substrate tracer, followed by assessment of label distribution in intermediates and product. A case in point is the discovery of the beta oxidation of long chain fatty acids by feeding animals phenylated long-chain fatty acids of different chain length. [37] At a more cellular level, the discovery of metabolic pathways by manometric methods has revealed an important principle still valid today: The importance of careful bookkeeping in experimental research. In biochemistry, the main "accounting tool", the

Warburg Manometer, owed its popularity for decades to the fact that it quantitated the interconnectedness of the metabolic pathways that underlie the flow of energy from substrates to ATP.

A more detailed study of the kinetic properties of individual enzymes, together with the development of simple, but specific enzymatic assays for intermediary metabolites, opened the way for the field of metabolic control analysis. [38] While investigators may be overwhelmed by the complex nature of metabolically-related data sets generated through 'omics', the chemistry involved in the individual steps of metabolic pathways is relatively simple: Consecutive steps in a pathway are related by the substrates and products of each individual reaction. However, the intersecting nature of metabolism requires that a number of compounds function both as intermediates and/or as precursors for more than one metabolic pathway (e.g., the glycolytic intermediate glucose 6-phosphate is a precursor for glycolysis, the pentose phosphate pathway, the hexosamine biosynthetic pathway, and glycogen synthesis). In this context it is important to distinguish between the terms <u>control</u> and <u>regulation</u> of metabolism [38]. Regulation occurs when a system maintains some variable constant in spite of fluctuating environmental conditions (= homeostasis). Control occurs when the system adjusts to match a change in demands. Accordingly, metabolic control is the power to change the state of metabolism in response to an external signal, e.g. an increase or decrease in blood supply or energy demand of the heart aiming at keeping the cellular ATP level constant (= "regulation").

<u>APPENDIX 5. Expanded Notes on General Methodological Considerations</u>

a) Rodent Strains

There are no expanded notes for this section.

b) Sex Differences

The National Institutes of Health (NIH) has recently issued a strict policy to emphasize the importance of studying both male and female animal models and cell lines.[39] Similar to the findings in humans, animal studies show male-female differences in many metabolic processes such as cardiac glucose uptake,[40] TG levels and turnover,[41] fatty acid metabolism,[42] and substrate selection for oxidation. For example, female mice lacking the peroxisome proliferator-activated receptor alpha (PPARα) have a survival advantage and less steatosis compared to their male counterparts.[42] This suggests that despite the lack of PPARα, female mice are better able to oxidize lipids.[42] Animal studies thus far have already shown the sexual dimorphism in cardiac metabolism is largely attributed to the effects of sex hormones, chromosomes, and sex-specific gene expression[43-50]. For example, estrogen-mediated male-female differences are documented in the heart and isolated cardiomyocytes for the expression of a number of genes, levels of miRNA, protein expression and post-translational modifications.[51] Whether phase of menstrual cycle by extension also affects cardiac metabolism is still a controversial issue.[43, 52] Sex and/or sex hormones also modify transcriptional coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) and (PPARα), [42] nitric oxide and PI3-kinase signaling, calcium handling, beta-adrenergic response, and contractile function. Lastly, male-female differences in cardiac metabolism may be mediated through changes occurring at another organ via secreted factors or changes in plasma metabolite levels. For example, to explain the sexual dimorphism in the heart's response to exercise, obesity, or diabetes, one needs to consider male-female differences in adipose tissue depot size, lipolysis, and plasma lipid delivery to the heart, which is a main determinant of myocardial substrate metabolism and function. Collectively,

these reports highlight the importance of considering biological sex as a variable in all human and animal study focusing on cardiac metabolism.

c) Circadian Considerations

Clearly, it is not feasible for the majority of investigational studies to be performed at multiple intervals over the course of the day. Prior to determining the optimal time-of-day to perform a particular assessment, one must consider: 1) available information (published or preliminary) regarding oscillations of the metabolic parameter of interest; 2) the hypothesized effect of the intervention; 3) for drug and/or dietary intervention studies, the time-of-day at which active agents influence the heart (and the rate of their turnover); and 4) similar considerations for the translation of basic observations to humans (e.g., the use of statins at bedtime). In the case of animal-based studies, should the optimal time of day appear to be during the dark (active) phase, use of altered light/dark cycle rooms within animal facilities enables completion of studies during normal working hours. Should the latter option be considered, adequate time needs to be given for equilibration of animals to the altered light/dark cycle (a minimum of one day for each hour shift). Use of red lights and night-vision goggles facilitates animal handling and simple procedures (*e.g.*, injections) during the dark phase. Importantly, the time at which a metabolic study is performed during the light/dark cycle should be reported within the Methods section of resultant manuscripts. It is noteworthy that due to the cell autonomous nature of circadian clocks, metabolic rhythms persist even in isolated cells (*e.g.*, cardiomyocytes), highlighting a need for similar considerations for *in vitro* studies.[53, 54]

Although "time" is mostly considered in terms of experiment duration (*e.g.*, time required for tracer equilibration, for achieving metabolic steady state *etc.*), or of the subject's age (*e.g.*, age/developmental stage at which a mouse model is investigated), time-of-day also needs to be

controlled for. Often this issue is side-stepped by consistently performing studies always at the same time of the day, influenced by practical convenience. However, this approach can be problematic, in terms of results obtained, conclusions drawn, and potential translation to the clinical setting. For example, laboratory rodents (a common animal model for cardiac metabolic and pathologic studies) are nocturnal animals, yet many exercise studies are performed during the light phase for convenience; such a study design is equivalent to exercise training humans in the middle of the night (*i.e.*, during the period of natural sleep). Similarly, fasting a rodent overnight (active phase) is a far greater metabolic stress compared to fasting a patient overnight (sleep phase).

d) Age

There are no expanded notes for this section.

APPENDIX 6. Expanded Notes on Radionuclide Tracers

a) Historical Notes

Following in the footsteps of Madame Curie,[55] George de Hevesy (1885-1966) was the first to use isotopic tracers to follow chemical reactions in the 1930s. De Hevesy was also the first to conduct experiments with radioactive isotopes on biological samples when he noted the uptake of radioactive lead by plants. Production of an unstable isotope of phosphorus (^{32}P) in 1934 enabled him to trace the movements of phosphorus in animal tissues.[56] Later he used heavy water ($^{2}H_2O$) to study transfer of water in goldfish (sic!) and, ultimately, in the human body.[56]

Around the same time Rudolf Schoenheimer (1898-1941) introduced the broader use of isotopic tracers into biochemical research in 1935. His use of isotope tracers, to study intermediary metabolism [57] changed the whole landscape of biochemistry. [58] Deuterium (heavy hydrogen) had become easily available for the first time, thanks mainly to the work of Harold Urey where Schoenheimer and Urey were colleagues at Columbia University. Schoenheimer used deuterium to replace some of the hydrogen of fat which he had fed to lab animals.[59] Until then it had been thought that fat stored in body tissues was immobile, just lying there until starvation demanded its use. On analyzing the body fat of rats several days after feeding them deuteriated fatty acids Schoenheimer observed that only about half the labeled fat was stored. He concluded that the other half of the fat must have been used and proposed that there was a rapid turnover of fat. In other words, body constituents far from being static, were changing constantly and dynamically.[57]

A few years later, when Urey had prepared the isotope nitrogen 15 (^{15}N), Schoenheimer used ^{15}N to label amino acids, the building blocks of proteins. In a series of experiments he fed single labeled amino acids to an animal, and traced the fate of that amino acid in the animal's proteins. Schoenheimer, again, found that there is constant action, even though the overall movement may be small, with the protein molecules constantly moving and shifting. Schoenheimer had thus established that many component molecules of the body are continuously broken down and built up. He summarized the finding in his short monograph *The Dynamic State of Body Constituents*,[60] which appeared a year after Schoenheimer's untimely death in 1941.

After World War II researchers led by Melvin Calvin (1911-1997) went on to use unstable radioactive isotopes, such as those of carbon (^{14}C) and hydrogen (^{3}H), to investigate

biochemical pathways first in plant cells and then in living animals.[61-63] While the principles were based on the pioneering work of Schoenheimer, the work with ^{14}C and ^{3}H proved the existence and function of the metabolic pathways constituting the intermediary metabolism of energy providing substrates and biosynthetic processes in the cell. Radioisotope studies proved the operation of metabolic pathways, including the citric acid cycle,[64] and continue to be widely used for the assessment of metabolic pathways and flux rates in cardiac metabolism.

b) Practical Considerations

Depending on the experimental system it is possible that incomplete metabolism of energy substrates may occur. For instance, incomplete oxidation of fatty acids can result in the accumulation of acid-soluble intermediates. This is especially true in preparations with a lower metabolic demand (i.e. isolated cardiomyocytes and heart homogenates). As a result, it is important to also follow the accumulation of radiolabelled acid soluble intermediates in these preparations.[65] Similarly, depending on the labeling pattern of the energy substrate, it is possible to over-estimate metabolic rates due to this incomplete metabolism. For instance, labeling of palmitate at carbon-1 with ^{14}C-, will release $^{14}CO_2$ following the first cycle of fatty acid ß-oxidation. Therefore, based on quantitative collection of $^{14}CO_2$ production, fatty acid oxidation rates are overestimated if incomplete oxidation of fatty acids has occurred. This problem is less relevant in the intact, pumping heart where higher metabolic rates result in very low incomplete oxidation rates.

Depending on the labeling pattern of the energy substrates it is possible to directly measure individual components of the energy metabolic pathways, such as flux through glycolysis, fatty acid oxidation or ketone body oxidation. However, in some situations labeling patterns do not allow the investigator to discriminate the exact source of metabolic by-product,

and a critical evaluation on the choice and use of glucose tracers is available.[66] For instance, uniformly labeling the carbon of glucose with ^{14}C (i.e. [U-^{14}C]glucose, does not allow discrimination of the $^{14}CO_2$ produced from flux through pyruvate dehydrogenase or the citric acid cycle. However, strategic labeling of pyruvate in the C_1 position (as 1-[^{14}C] pyruvate) allows for assessment of pyruvate carboxylation and anaplerosis of the citric acid cycle.[67]

APPENDIX 7. Expanded Notes on PET/ SPECT

a) Assessment of Metabolic Activity In the Heart *in vivo*

The development of the radionuclide imaging to measure myocardial metabolism has been shaped by several key efforts over the past century. Its birth can, again, be traced to the 1920s when George Hevesy introduced the concept of radiotracers to study cellular metabolism in living systems.[68] With respect to imaging systems, key advances occurred in the 1950s to early 1960s with the introduction of the concept of emission and transmission tomography and the first demonstration of annihilation radiation for medical imaging.[69, 70] The application of these concepts led to the development of the first PET systems in the 1970s.[71] These efforts culminated in the introduction of hybrid systems such as PET/CT in the early 2000s and more recently, PET/MR, permitting the integration of measurements of myocardial metabolism with those of left ventricular structure and function. From a radiopharmaceutical development perspective, the first cyclotron for biomedical applications was introduced in the late 1930s. This ignited the use of PET and oxygen-15 to measure cellular oxidative metabolism. In the 1970s and 1980s the development and implementation of radiopharmaceutical synthesis schemes permitted the radiolabeling of naturally occurring substrates such as glucose and palmitate with carbon-11 and substrate analogs such as deoxyglucose, with fluorine-18.[72] Developed in parallel

were image analysis schemes that permitted the quantification of key myocardial metabolic processes such as glucose uptake, fatty acid uptake and oxidation and oxygen consumption in humans.[73] The availability of these techniques set the stage for the non-invasive regional assessment of cardiac metabolism in a host of normal and abnormal pathophysiologic conditions, including seminal studies demonstrating the assessment of myocardial glucose metabolism as a marker of tissue viability.[74] Examples of pre-clinical and human applications of metabolic imaging are in the **Appendix** (**Table A2**).

b) Further Developments

Despite the success of metabolic imaging from both a research and clinical perspective, advances are needed in several areas. Advances in PET detector design and post-detector electronics are expected to increase counting statistics which would improve the ability to perform more complex compartmental modeling permitting better characterization of the metabolism of a given substrate. New designs in SPECT technology may permit dynamic data acquisitions allowing quantitative or semi-quantitative measurements of substrate metabolism. New radiotracers are under development that enable characterization of key metabolic pathways such as uptake, storage or oxidation of specific substrate molecules that are linked to disease manifestations as well as provide insights into the linkage between substrate metabolism and cell growth, cell survival and energy transfer. To facilitate their widespread usage, these radiotracers will need to be radiolabeled with F-18, I-123 or Tc-99m and exhibit kinetics that can be assessed with readily exportable image analysis schemes. Such advancements will facilitate the performance of appropriately powered clinical trials designed to answer questions about the utility of metabolic imaging for diagnosis, risk stratification and monitoring of treatment response in specific patient populations.

APPENDIX 8. Expanded Notes on ^{31}P and ^{1}H-MRS (NMR)

a) Historical Notes

The second half of the 20th Century has witnessed the development of nuclear magnetic resonance, or NMR, spectroscopy (also termed magnetic resonance spectroscopy, MRS) into a major tool for analytical chemistry and materials science, a technique in structural biology, an imaging procedure in diagnostic radiology, and a window on metabolism in living cells and organs.[75] The terms NMR spectroscopy and MRS are used interchangeably.

MRS has become one of the standard methods in experimental cardiology, since the first ^{31}P-MR spectrum of an isolated heart was obtained by Garlick, Seeley, and Radda, in 1977.[76] Since then, MRS has been widely used for the study of many aspects of cardiac metabolism. In addition to Radda's group in Oxford, other early groups in the field were those led by J.S. Ingwall[77] and W.E. Jacobus.[78] Almost at the same time Balaban and his group became the first to study the relationship between cardiac energetics and workload in large animals,[79] followed by Zhang and Ugurbil's work on the left ventricular transmural gradient of cardiac energetics at rest and stress.[80] In the 1990s, Ingwall's group introduced the use of MRS in genetically manipulated mice.[81] Many of the methodological contributions on human cardiac ^{31}P-MRS come from the work of Bottomley and Weiss,[82-84] initially developed at 1.5Tesla. Using one-dimensional ^{31}P-NMR spectroscopic and stacked plot analyses Conway et al were the first to detect a low PCr/ATP ratio in failing human heart *in vivo*.[85] Neubauer's group first demonstrated that cardiac MRS measurements of energetics were predictors of prognosis for patients with heart failure.[86] Most of the literature on human cardiac ^{1}H-MRS of lipid changes has been contributed over the years by Szczepaniak's group in Dallas and by Lamb's group in

the Netherlands.[87, 88] More recently, Robson's group has pioneered human cardiac MRS at higher field strength such as 3 and 7 Tesla.[89, 90]

b) Experimental Applications of ^{31}P-MRS

^{31}P-MRS has been used in experimental cardiology for nearly 40 years.[91] The basic principles of MRS are best illustrated in the most widely used model, the saline perfused rodent heart. The heart is studied inside an MR spectrometer, requiring a high-field superconducting magnet (currently up to 18 Tesla, the unit of magnetic flux density). The radiofrequency (RF) coils, which are used for MR excitation and signal reception, are seated in the magnet bore. A computer, a magnetic field gradient system, and a radiofrequency (RF) transmitter, and a receiver are also needed. First, the magnetic field is homogenized with shim coils to balance out small field in homogeneities for optimal spectral or image quality. A radiofrequency pulse, sent by the RF coil(s), excites the nuclear spins in the tissue. The MR signal, the free induction decay (FID), is then recorded and "Fourier transformed", which results in an MR spectrum, showing resonance frequency on the x-axis and signal intensity on the y-axis. Due to the faintness of the MRS signal, hundreds of FIDs have to be averaged to obtain a sufficient "signal-to-noise ratio" (SNR; the signal amplitude divided by the standard deviation of noise). For a perfused rat heart experiment at a magnetic field strength of >7 Tesla, a ^{31}P-MR spectrum is typically acquired in ~5 mins. Spectra need to be corrected for the effects of partial saturation, because ^{31}P resonances have different T1 relaxation times (e.g. PCr T1 is longer than that of ATP).

c) Clinical Applications of ^{31}P-MRS

Human cardiac MRS has been performed on 1.5T, 3T, and, most recently, on 7T MR systems.[92-94] Compared to standard MRI systems, additional hardware requirements are a broadband RF generator and a ^{31}P surface coil (single coil or array). Additional software, i.e. ^{31}P-acquisition sequences, is also necessary. The strong ^{31}P-signal from chest wall skeletal muscle must be suppressed by spectral localization methods, such as DRESS (**d**epth-**re**solved **s**urface coil **s**pectroscopy), rotating frame, 1D-CSI (**c**hemical **s**hift **i**maging), ISIS (**i**mage-**s**elected ***in vivo*** **s**pectroscopy), and 3D-CSI.[93] Most MRS studies are performed in a prone position, rather than supine, because this reduces motion artifacts and the distance of the heart from the surface coil, thereby improving sensitivity. The majority of spectroscopic techniques require a stack of ^{1}H scout images to be obtained first, which are used to select the spectroscopic volume(s); however, the spectral localization techniques lead to a loss of SNR, and up to 10% of the signal may still arise from outside the selected voxel. Saturation bands that suppress the chest wall skeletal muscle signal substantially reduce this contamination. At 1.5 and 3T, human cardiac ^{31}P-spectra can be obtained in ~10-30 mins from 20-70ml voxels. A ^{31}P-MR spectrum of a healthy volunteer obtained with 3D-CSI is shown in (**Figure A1)**. Two additional resonances are detected: 2,3-diphosphoglycerate (2,3-DPG), arising from erythrocytes in the voxel, and phosphodiesters (PDE), a signal from membrane and serum phospholipids. The 2,3-DPG peaks overlap with the Pi resonance, which therefore cannot be detected in human ^{31}P-MR spectra containing blood signals. Thus, intracellular pH can also not be determined. However, Pi and pH should become detectable in human myocardium when spatial resolution is increased to minimize blood contamination of ^{31}P-spectra. By calculating the PCr/ATP and PDE/ATP peak area ratios, relative quantification of human ^{31}P-spectra is straightforward. PCr/ATP is used as an index of the energetic state of the heart, while the relevance of the PDE/ATP ratio remains unclear. In

addition to saturation (see above), ^{31}P-spectra require correction for blood contamination: Blood contributes signal to the ATP-, 2,3-DPG- and PDE-resonances. As human blood spectra have an ATP/2,3-DPG area ratio of ~0.11 and a PDE/2,3-DPG area ratio of ~0.19, for blood correction, the ATP resonance area of cardiac spectra is reduced by 11% of the 2,3-DPG resonance area, and the PDE resonance area is reduced by 19 % of the 2,3-DPG resonance area. [1] However, 2,3-DPG levels in erythrocytes may be variable. Absolute quantification of PCr and ATP is technically challenging, but is desirable, as the PCr/ATP ratio does not detect simultaneous decreases of both PCr and ATP, which occur in the failing[95] or in infarcted non-viable tissue. Absolute ^{31}P-metabolite levels can be obtained by acquiring signal from a ^{31}P-standard as well as estimates of myocardial mass from MRI.[93] Alternatively, the ^{31}P-signal can be calibrated to the tissue water proton content, measured by ^{1}H-MRS. [93] Lastly, the signal-to-noise ratio (SNR) increases linearly with field strength and the advent of 7T human MRI systems provides this advantage. Recent work shows that SNR of cardiac ^{31}P-MRS is increased 2.3- and 5-fold compared to 3 and 1.5T, respectively.[96] The creatine kinase (CK) reaction velocity can be measured in human heart in ~30 mins with the "four-angle saturation transfer" (FAST) method, although this has so far only been reported by one group,[97] and has not been verified by others.

APPENDIX 9. Expanded Notes on ^{13}C-MRS (NMR)

a) Assessment of Cardiac Metabolic Activity by ^{13}C-NMR Spectroscopy

The aim of probing cardiac metabolism with the stable isotope ^{13}C is to assess metabolic efficiency through shifts in the oxidation of carbon-based fuel sources for ATP production. Regulation of metabolic flux and rates of oxidative metabolism at the enzymatic and genomic level, and activity shifts among competing pathways all can contribute to metabolic signaling

mechanisms that mediate the cardiac responses to pathological stress, including nutrient/metabolic stress, on the heart.

^{13}C NMR, PET and SPECT are clinical methods suitable for imaging a single energy-providing substrate, while the use of radiotracers such as ^{3}H or ^{14}C have a long history in research studies of isolated tissues and the human hearts. However, neither PET nor SPECT provide information about the chemical state of the tracer. Also, while ^{3}H released in the form of $^{3}H_2O$, or ^{14}C released in the form of $^{14}CO_2$ provide utilization rates of the parent compound, clinically these methods can only be performed in the cath lab. Although ^{13}C NMR methods generally require significant amounts of isotope enriched material and have the potential to influence the metabolism under study, a major advantage of the stable isotope method is the utility to provide data on both enriched and non-enriched metabolites, with follow up experiments that combine detection by either *in vitro* NMR or mass spectrometry to determine fractions of ^{13}C and endogenous ^{12}C components in the metabolite pools of interest.

A practical consideration for ^{13}C NMR detection is the need to simplify resonance signal peaks within spectra that are otherwise influenced by the presence of covalent bonding to hydrogen. While spin-spin coupling between ^{13}C nuclei is advantageous for detailing patterns of substrate oxidation in the heart via delivery of exogenous ^{13}C-enriched fuels, the resonance signals emanating from ^{13}C are split by spin-spin coupling with the many protons (^{1}H), also possessing net spin, that are covalently bonded as hydrogen to ^{13}C nuclei in biological compounds. The resulting ^{13}C-^{1}H coupling produces many overlapping multiplets within the individual resonance signals of all the ^{13}C nuclei that complicate ^{13}C NMR spectra and can confound analysis. The complexity is remedied by applying a second radiofrequency at the resonance frequency of ^{1}H, a process called proton decoupling that imparts energy to the ^{1}H

nuclei to maintain a "saturated" spin state. In this state, the transitions between energy levels of the excited ^{1}H nuclei are sufficiently rapid to be averaged out, as are the imparted energy states on any coupled nuclei. Therefore, application of a second resonance frequency collapses the multiplet structures of the NMR "peaks" from the ^{1}H -coupled ^{13}C nuclei, simplifying interpretation of the ^{13}C NMR data set. Though technically easy to apply, attention to sample heating is required due to the RF energy deposition that induces heating, especially of live samples that must be maintained at 37°C.

The effect of decoupling, in collapsing the multiplet structures of ^{13}C resonance signals that occur through ^{13}C-^{1}H coupling, also serves the purpose of increasing the available peak height above baseline. This is a particularly important component for studies performed in intact organs where lines are generally broadened due to less homogeneous magnetic fields in the beating heart and where signal can be low due to requirements for short data collection times. Another advantage of proton decoupling is the inherent augmentation in the NMR signal produced in the ^{13}C observed NMR spectrum due to energy transfer from the excited protons to the bonded ^{13}C nuclei. Dipole relaxation mechanisms in the proton population that is excited by the second, decoupling frequency impart additional energy to the covalently bonded ^{13}C nuclei, resulting in a significant increase in the signal intensity of the proton decoupled, ^{13}C NMR spectrum. This process is called Nuclear Overhauser Enhancement, or NOE, and theoretically enhances the ^{13}C NMR signal two-fold, although in biological systems it is slightly less.[98] Nonetheless, conventional ^{13}C NMR detection enables metabolic assessment of the heart with precision. It offers a very unique application to assess shifts in the oxidation of various fuels by the heart, actual rates of metabolic flux through the oxidative pathways, substrate sharing among

competing pathways, and even the rates of transport dependent processes across the sarcolemma and mitochondrial membranes.

As already mentioned elsewhere, the heart has the capacity to switch rapidly among energy-providing substrates depending on the heart's environment. Thus, unlike the brain where under normal conditions energy is derived from glucose oxidation, the heart readily switches substrates among fatty acids, glucose, ketone bodies, lactate and other substrates depending on their availability, the neurohumoral, environmental, and the hemodynamic load.[7, 99, 100] Shifts among these metabolic pathways also generate by-products that induce metabolic signaling mechanisms in the cytosol, the mitochondria, and the nucleus.[101, 102] Hence, the challenge is how to monitor simultaneously the contributions of fatty acids, ketone bodies and carbohydrates.[7, 99-101, 103, 104]

^{13}C NMR methods capitalize on the fact that acetyl-CoA enters the citric acid cycle as the final common pathway for oxidation of all substrates and some intermediates of the Krebs cycle (or exchanging amino acids) are easily monitored. Because the acetyl moiety contains two carbons, there are 4 possible isotopomers of the acetyl group.[105, 106] For this reason, specific ^{13}C labeling in the parent molecule will yield distinct labeling in acetyl-CoA. For example, [U-^{13}C] palmitate can only generate [1,2-^{13}C]acetyl-CoA, [1,6-^{13}C] glucose can only generate [2-^{13}C]acetyl-CoA, and [1,3-^{13}C]acetoacetate can only generate [1-^{13}C]acetyl-CoA. Unlabeled glycogen or TG yield unenriched acetyl-CoA. If one could determine the relative concentration of each of the 4 acetyl-CoA isotopomers, then it would also be a simple matter to determine the fraction of energy provision from glucose, fatty acids, ketone bodies and endogenous sources. This information is provided by ^{13}C NMR observation of glutamate (See **Figure 5** in the main text of the Scientific Statement).

The distribution of ^{13}C glutamate isotopomers can be elucidated from the relative intensity of the multiplet structures within the ^{13}C NMR signals from glutamate carbons. Although the low natural abundance of ^{13}C limits detection, this feature is actually a major advantage, because being a dilute spin, the chance of having one ^{13}C covalently bonded to another ^{13}C is extremely low (0.011 x 0.011 = 0.00012). As a result, the appearance of adjacent ^{13}C nuclei can for practical purposes only arise from administration of an enriched compound followed by its metabolism. [107] Although not an instantaneous readout, because the interconversion of α-ketoglutarate and glutamate is a rate determining process that can become disassociated with citric acid cycle flux, the analysis of substrate selection from glutamate isotopomers has sufficient internal standards to be independent of both rates and steady state conditions, which is especially useful for heart studies[108-112] **(Figure 5** in the main text of the Scientific Statement**).** The term "isotopomer" arises from the contraction of "isotope" + "isomer", and refers to the distinct distribution of ^{13}C in otherwise identical molecules.[113] For example, glutamate with ^{13}C in positions 3 and 5 is identified as [3,5-^{13}C] glutamate, and glutamate labeled in position 3 and 4 is identified as [3,4-^{13}C] glutamate. Although both molecules have 2 extra ^{13}Cs, the labeling pattern is different. Depending on the situation, specific isotopomers or groups of isotopomers are easily identified from the ^{13}C NMR spectrum due to the information provided by chemical shift and spin-spin coupling.

This information arises because the chemical shift of carbon 4 of glutamate is distinctive, and the presence (or absence) of ^{13}C in the adjacent carbon 5 is detected by the presence (or absence) of the characteristic C4-C5 spin-spin coupling. In this example, any glutamate enriched in both positions 4 and 5 can only arise from [1,2-^{13}C]acetyl-CoA which in turn is generated exclusively from the fatty acid (in this case palmitate). Similarly, glutamate labeled with ^{13}C in

the position 4 but not in position 5 can only arise from [2-^{13}C]acetyl-CoA which, in turn, is derived exclusively from [1,6-$^{13}C_2$]glucose. Hence, it is easy to measure the relative oxidation of glucose and fatty acids, and, by extension, the relative contribution of ketone bodies and of unlabeled substrates. This analysis does not require mathematical models or any metabolic or isotopic steady states, and consequently can be used under shifting metabolic conditions such as ischemia and reperfusion.[103, 108] Because of the poor NMR sensitivity, these methods are largely restricted to studies of tissues that have been freeze-clamped, followed by extraction and analysis in a standard analytical NMR instrument.[106]

For organic compounds not readily oxidized, administration of ^{13}C enriched substrate will often not produce sufficient NMR signal to detect the multiplet structures, precluding determination of glutamate isotopomers for any calculation of the fractional enrichment of acetyl-CoA. This becomes a particular problem for studies in the *in vivo* heart. In experimental comparisons of the relative amount of substrate oxidation can be reliably implemented by examining the fractional enrichment of the primary site of carbon labeling within glutamate in a tissue extract, i.e. 4-carbon or 5-carbon depending on the original site of ^{13}C enrichment of the substrate.[114, 115] The fractional enrichment of glutamate can be simply determined by comparison of the NMR signal intensity from the experimental sample to that of an external concentration standard of glutamate, often 100 mM glutamate, which contributes NMR signal from the 1.1 mM naturally abundant ^{13}C at each carbon position.[105, 115, 116]

Isolated heart studies allow metabolite detection at comparatively high magnetic field strengths ranging from 9.4 to 14.1 Tesla. Kinetic measurements of metabolic flux from NMR-detected rates of ^{13}C enrichment in the heart were originally limited to studies employing acetate or butyrate. More recently, the availability of specific ^{13}C enrichment patterns within long chain

fatty acids, the preferred fuel in the heart, has led to a more realistic assessment of metabolic regulation in the heart.[101, 117-120] With the understanding of the dynamic, rate limiting processes that contribute to the progressive enrichment of glutamate, ^{13}C NMR permits examination of the relationship of Krebs cycle flux to the oxidation rates of individual substrates, the regulation of the reciprocal processes of β-oxidation and TG synthesis and lipolysis, and the balance between citric acid cycle generation of NADH and the contribution of cytosolic NADH to oxidative ATP production.[101, 120, 121]

The evaluation of metabolic activity, including flux measurements, in the heart is reliant on the detection of ^{13}C-enriched glutamate, mainly due to low intracellular content of the Krebs cycle intermediate, as discussed above. Once assumed to be in rapid exchange with ^{13}C enriched α-ketoglutarate, we now know that glutamate enrichment is dependent on both the rate of enrichment of α-ketoglutarate and on the rate of α-ketoglutarate oxidation via α-ketoglutarate dehydrogenase within the Krebs cycle and the competing process of mitochondrial α-ketoglutarate efflux of to the cytosol, where the bulk of NMR detectable glutamate is located.[110-112, 121-125] (**Figure 5** in the main text of the Scientific Statement**).** Adaptive and maladaptive states of the heart heavily influence this balance, and, consequently, the interconversion rate between α-ketoglutarate and glutamate can be temporally disassociated from Krebs cycle flux and the rate of α-ketoglutarate enrichment. Indeed, at very high workloads in the *in vivo* heart, glutamate enrichment with ^{13}C from the Krebs cycle can be completely bypassed due to the high rate of oxidation that outcompetes the interconversion of α-ketoglutarate to glutamate.[112]

Site-selective ^{13}C enrichment of LCFA can offer simplicity in the analysis to enhance the information content and accuracy of protocols measuring rates of fatty acid oxidation in the intact beating heart. While such labeling schemes have great utility for specific applications, the

synthesis of "designer" LCFA with ^{13}C at specific carbon sites, is costly in comparison to the entirely enriched, U-^{13}C LCFA.

b) Expanded Notes on Hyperpolarized ^{13}C NMR

NMR sensitivity is controlled in large part by the Boltzmann distribution. This relationship indicates that at body temperatures the NMR sensitivity of ^{13}C is poor because the difference in ^{13}C populations in the two possible polarization states ("up" vs. "down" relative to the external magnetic field) is very small, a few parts per million at most. By acquiring data from concentrated samples at high magnetic fields over prolonged periods, the sensitivity constraints of ^{13}C NMR are overcome. Hyperpolarized [1-^{13}C] pyruvate serves as a particularly convenient metabolic probe for analysis of cardiac metabolism, because the T1 nuclear relaxation time, the rate of loss of hyperpolarized magnetization, is relatively long. However, as discussed elsewhere, the high concentrations of pyruvate required and the changing blood levels following bolus injection may perturb the physiological state and present challenges to quantitative evaluations of metabolism. Longer T1s simplify technical aspects of these studies, and other probes such as [1-^{13}C] acetate also provide different types of information about oxidative metabolism.[126]

While the above approach is useful for experimental work, it cannot be used for clinical imaging as yet. With an early focus was on cancer,[127] the first *in vivo* cardiac studies in the pig[128, 129] were followed quickly by studies in isolated rat hearts,[130, 131] and the rat heart *in vivo*.[126, 132] An important technical advance was the demonstration of multi-slice ^{13}C imaging in the pig heart.[133] As a result of this rapid progress plus the recent demonstration of safely imaging hyperpolarized pyruvate and metabolic products in patients with prostate cancer,[134] there is interest in rapid translation to human patients with heart disease.[135, 136]

At present, most analysis of cardiac metabolism using hyperpolarized ^{13}C utilizes delivery of exogenous [1-^{13}C] pyruvate, yet other compounds of physiological relevance are on the horizon. Pyruvate may undergo decarboxylation to form hyperpolarized $^{13}CO_2$ and bicarbonate[137] and, consequently, provide a direct index of mitochondrial integrity. Pyruvate is also carboxylated, transaminated to form alanine, or reduced to form lactate.[138-140] The relative rate of metabolism of hyperpolarized pyruvate to CO_2 compared to lactate may prove to be a useful indicator of preserved mitochondrial function. Hence, at least two types of information become available: 1) pyruvate transport and metabolism in the cytosol from the lactate and alanine images, and 2) pyruvate oxidation in the mitochondria. Pyruvate carboxylation occurs either by malic enzyme or by pyruvate carboxylase.[18, 67] Early clinically relevant models have investigated myocardial infarction[128] and heart failure.[141]

In practical terms, hyperpolarized materials must be injected in high concentrations so the tracer itself may actually alter metabolism transiently, and the mass of injected material must be evaluated for safety. High pyruvate concentrations are, however, not physiological and activate the pyruvate dehydrogenase complex.[142] Polarizers suitable for human studies must provide sufficient mass of material and assure quality of the injected material, such as sterility. Because these devices are not generally available, and are expensive, human cardiac studies have not yet been performed. Lastly, although there are fundamental advantages of ^{13}C methods, comparison to long-accepted technologies still needs to be carried out.

c) Expanded Notes on Quantitating Metabolic Flux Rates in Isolated Hearts with Conventional ^{13}C NMR Detection

Following NMR detection of the rates of glutamate enrichment, experimental determination of the relevant citric acid cycle intermediate content of the heart and the fractional

^{13}C enrichment of acetyl CoA, from *ex vitro* NMR of the glutamate isotopomer distribution, are combined into a compartment model that can be described by a set of simple, linear differential equations. Rates are then determined by least square fitting of the metabolite input to the glutamate enrichment curves.[109, 110, 121, 123, 143]

The additional consideration of competition between α-ketoglutarate oxidation and exchange with malate in assessing the rates of oxidative metabolism in the heart is an opportunity to gain further insights into metabolic activity of the heart. The rate of glutamate ^{13}C enrichment is influenced by both, the Ca^{2+}-dependent component, and the affinity of the α-ketoglutarate dehydrogenase enzyme for its substrate as this enzyme competes with OMC for the common substrate, α-ketoglutarate.[110, 111] The malate exchange process through OMC is part of the malate-aspartate shuttle system which supports transfer of cytosolic reducing equivalents produced from glycolysis to enter the mitochondria and supply the electron transport chain. Therefore, changes in OMC activity can be reflected in the rates of isotopic enrichment of the carbon positions of glutamate for study of cytosolic NADH/NAD^+ regulation and the metabolic linkages between the cytosol and mitochondria.[111, 120, 144]

d) Expanded Notes on Rates of Fatty Acid Oxidation and Storage

As described earlier, detection of ^{13}C enrichment rates of the glutamate 4-carbon relative to subsequent enrichment rates of the 2- or 3-carbons is fundamental to the approach. Fully labeled LCFA, U-$^{13}C_{16}$ palmitate or U-$^{13}C_{18}$ oleate, enable progressive ^{13}C enrichment of TG to be monitored by sequential ^{13}C NMR spectra. ^{13}C-^{13}C spin coupling between carbon positions that split the signal serve to reduce the vertical amplitude of signal for detection of glutamate resonances above baseline noise. Thus, ^{13}C enrichment of the LCFA at only the even-numbered carbon positions of the LCFA chain will reduce the signal splitting and enable maximum vertical

scaling for signal detection. However, analyzing oxidation of ^{13}C enriched LCFA can be further complicated by overlapping NMR signal from ^{13}C LCFA that may confound detection of the glutamate 4-carbon signal. Eliminating enrichment at the 2-carbon of ^{13}C LCFA, such as [4,6,8,10,12,14,16-$^{13}C_7$] palmitate or [4,6,8,10,12,14,16,18-$^{13}C_8$] oleate, resolves potentially confounding kinetics between glutamate 4-carbon signal and LCFA 2-carbon signals.[145]

APPENDIX 11. Expanded Notes on Turnover of Intracellular Macromolecules

a) Historical Notes

The concept of a "dynamic state of body constituents" was first enunciated by Schoenheimer who followed the fate of the stable isotopes ^{2}H and ^{15}N in the mammalian body.[60] Schoenheimer was the first to provide clear experimental evidence of the dynamic concept of metabolism when he worked with Harold Urey at Columbia University in New York, who was an expert in the separation of isotopes. Through Urey the heavy isotopes deuterium and ^{15}N became available for biological experimentation.[146] In the short monograph "The Dynamic State of Body Constitutents", based on The Edward K. Dunham Lectures for the Promotion of the Medical Sciences at Harvard University in 1941,[60] Schoenheimer writes: "*The finding of the rapid molecular regeneration, involving constant transfer of specific groups, suggests that the biological system represents one great cycle of closely limited chemical reactions",* and he continues; "*The new results imply that not only the fuel, but (also) the structural materials are in a steady state of flux. The classical picture must thus be replaced by one which takes account of the dynamic state of body structure*." Seventy years on heavy water ($^{2}H_2O$) is used for the assessment of cardiac proteome dynamics.[147] and for the interrogation of *in vivo* protein turnover in the heart.[148]

b) Assessing Redox Active Metalloproteins and Cardiovascular Disease.

The central of transition metals, particularly iron and copper, as co-factors in a broad range of proteins involved in many aspects of biology including signaling and metabolism underlies their important role in the pathophysiology of cardiovascular disease. Here we will focus on one of the best known examples; the heme prosthetic group which incorporates iron into proteins in a way that allows for an exquisite selectivity in function including selectivity in ligand binding or electron transfer. This is particularly striking in the ability of the cell to discriminate between nitric oxide, carbon monoxide and oxygen by utilizing the local environment of the heme prosthetic group. This is nicely illustrated in the context of nitric oxide signaling and its role in physiology and pathology[149, 150]. The primary heme-dependent sensors for nitric oxide are soluble guanylate cyclase and the mitochondrial terminal electron acceptor, cytochrome *c* oxidase[151, 152]. Oxygen can be reduced to superoxide at the active site of nitric oxide synthases in a process in which nitric oxide synthesis is "uncoupled" from NAPDH oxidation and is a hallmark of cardiovascular dysfunction[153]. In the case of cytochrome *c* oxidase the opposite situation prevails where oxygen is the preferred ligand and NO is a competitive inhibitor and so modulates respiration. New properties for the heme-dependent reductive pathways continue to emerge including the conversion of nitrite to nitric oxide under conditions of hypoxia[154]. The newest member of the heme ligands is hydrogen sulfide which is rapidly emerging as a novel mediator of the cross-talk between protein thiol and metalloprotein signaling[155]. Not surprisingly, under conditions where heme is lost from the protein its hydrophobic nature results in incorporation into membranes. In this environment the redox reactions of the iron are uncontrolled and can promote an intense pro-oxidant reaction which causes bioenergetic dysfunction, autophagy and when these protective pathways fail damages the

endothelium[156]. Interestingly, heme is highly reactive causing protein oxidation in the extracellular environment, forms adducts with lipoproteins and contributes to oxidative stress surrounding the heart following surgery[157, 158].

c) Dynamics of Myocardial Protein Turnover

The dynamics of incorporation of labeled amino acids into the protein pool over time in the steady state and their release can be described by the following differential equation[159]:

$$1) \quad dP^*/dt = K_s F^* - K_d P$$

in which P* is the specific activity of a labeled amino acid in the protein product pool (P) and F* is the specific activity of a labeled amino acid in the free amino acid pool. The term "specific activity" refers to the ratio of the amount of radioactivity in the amino acid to the total amount of amino acid in the precursor and product pools, expressed as dpm/nmol. The incorporation and release of the tracer into the pools are defined by first-order rate constants, K_d and K_s. Hence, protein synthesis and degradation are represented through these rate constants. The amino acid used in this precursor-product analysis has to fulfill certain criteria to accurately determine protein turnover rates. Specifically, the tracer amino acid must be taken up into the cell and incorporated into the protein with the same rate as the unlabeled amino acid. The intracellular concentration of the amino acid needs to be stable and have a detectable rate of incorporation into proteins without disturbing the cellular system as a whole.

The most rigorous approach to measure rates of protein synthesis, either *in vivo* or *ex vivo,* is to measure directly the specific radioactivity or the abundance of the tracer amino acid bound to tRNA in the course of biosynthetic labeling.[160] (See **Figure 7**) Rapid i.v. injection of a radiolabeled amino acid, along with a massive amount of the same unlabeled amino acid may

obviate the need to isolate the true protein precursor (aminoacyl-tRNA) because of the assumption that the aminoacyl-tRNA pool of the injected amino acid immediately equivocates with the expanded tissue and plasma pools.[160]

The radioactive labeling methods do not provide a direct estimate of protein degradation. The precise determination for a direct estimation of the rate of degradation of individual proteins remains elusive, although refined stable isotope methods coupled to mass spectrometry are providing new insights.[147, 148, 161] In a steady state system of myocardial protein synthesis and degradation are (by definition) equal. Thus, the fractional rate of protein degradation (K_d) is equal to the fractional rate of protein synthesis (K_s). Based on a simple one-compartment model for protein degradation the half-life of an individual protein ($T_{½}$) is inversely proportional to K_d:

$$2) \quad T½ = \frac{\ln 2}{Kd}$$

Of note is that the rates of protein degradation expressed in tens of protein half-lives require steady state systems demonstrating random decay.[160] In non-steady state conditions K_d may be either smaller or larger than K_s (although other permutations are possible.)[162]

A different radioactive tracer method to assess protein turnover of the heart involves the extraction of intravenously infused radiolabeled phenylalanine and the dilution of its specific activity across the heart at isotopic steady state. Using arterial and coronary sinus catheterization and a continuous infusion of radiolabeled phenylalanine, the uptake of tracer ("hot") phenylalanine from plasma and the release of unlabeled ("cold") phenylalanine are measured.[163] In conjunction with a model that assumes rapid equilibration of tracer specific activity between myocardial phenylalanyl – tRNA and circulating phenylalanine rates of myocardial protein synthesis and degradation can be estimated.[163, 164] This method has been verified by the

incorporation of radioactive phenylalanine into myocardial protein[163] and has discovered the anabolic effects of branched chain amino acids on the human heart.[165]

d) Assessing Triacylglycerol (TG)Turnover: **New Insights**

New insights have been gained from NMR based detection of multi-phasic components of TG enrichment with ^{13}C from administration of ^{13}C LCFA. The approach has elucidated separate kinetic signatures that are associated with transport- mediated LCFA uptake into the cardiomyocyte or the synthase and lipase activities that determine turnover of LCFA within the TG pool.[101, 166, 167] With sufficient signal-to-noise within the ^{13}C NMR spectra that will allow for the necessary temporal resolution, typically 1-2 min per spectrum, the isotope enrichment kinetics of triacylglycerol in the heart can be resolved into two distinct components: An initial, fast exponential and a slow, apparent linear phase, that, in actuality, is the rise component of a slower exponential that would otherwise require 5-6 hours to reach steady state.[167] The initial, fast exponential is specific to the uptake of LCFA into the cardiomyocyte via transport across the sarcolemma,[167] while the apparent linear phase is specific to turnover within the TG pool.[101, 167] To assess changes in LCFA uptake from the initial exponential, the time constant or rate constant of the initial exponential phase, can be calculated using the following equation:

$$Y=TGss-(TGss-TGo)\, e^{(-k\,(X-X_o))}$$

Where, TGss = enriched TG at steady state; TGo = enriched TG at time 0 (should be 0, but in some experiments this might not be the case); k = rate constant; X = time; Y = enriched TG level at time X; 1/k = time constant

While the actual processes associated with these separate kinetic features occur continuously and simultaneously, NMR detection during the initial introduction of isotope

elucidates and distinguishes these components in a temporal fashion. With end point quantification of total TG and the fractional ^{13}C enrichment of the acyl groups, the slope of the linear phase provides the TG synthesis rate of all acyl groups, ^{13}C enriched and unenriched, ^{12}C LCFA. As mentioned above, with steady state concentration of TG throughout the detection and collection protocols, rates of synthesis equal rates of degradation and provide a measure of total TG turnover. Examination of endogenous TG dynamics in the heart has provided unexpected, new insights into the role of the stored lipid pool as a physiologically active component of cardiac metabolism. Examinations of TG synthesis and lipolysis in the diseased heart are beginning to unveil the role of lipid storage dynamics on the activation state of energy providing pathways, as both a fuel source for oxidative metabolism and as a source of metabolic signaling within the cardiomyocyte that responds to metabolic stresses.

APPENDIX 12. Expanded Notes on Probing Cardiac Metabolism *in vivo*

a) Historical Notes

Work on metabolism of the human heart and the dog heart *in vivo* began with the investigations by Richard Bing and his colleagues.[168, 169] Their studies were the result of two major technical advances: 1) the development of a technique for coronary sinus catheterization and its application to the human heart,[170] which made it possible to sample blood from the venous bed of the heart; and 2) improved analytical methods which enabled more accurate and reproducible estimates of glucose, lactate, pyruvate, fatty acid, ketone body, and amino acid utilizaton, as well as of myocardial oxygen consumption. With those methods Bing and his lab were able to show, either directly or indirectly, that the human heart uses the three foodstuffs carbohydrates, fatty acids and (under certain circumstances also) amino acids to varying degrees for its energy provision.[168, 169]

Bing's work suggested that the arterial concentration of carbohydrates affects their relative myocardial usage.[168] However, they noted that the total aerobic metabolism of glucose, lactate, and pyruvate fell short of the total oxygen consumption of the heart, and it was quickly learned that oxidation of fatty acids and ketone bodies made up for the large (70%) balance. Subsequent to this initial observation Bing's work firmly established the preferential oxidation of fatty acids by the heart in the fasted state.[169] Myocardial utilization of fatty acids and also of TG was particularly high in the fasted state after a high fat meal. Bing also learned that, in normal heart the oxidation of ketone bodies accounted for approximately 5% of the total myocardial oxygen consumption, but when dogs were made diabetic, the dog heart utilized a consistently larger quantity of ketone bodies.[171] The utilization of ketone bodies was governed by their arterial concentration and by the quantity of carbohydrate available.[169] Bing's work described, as well, that the human heart (and the dog heart) *in vivo* extracted considerable amounts of amino acids from the blood when arterial concentrations were increased.[169] After infusion of a mixture of amino acids and an increase in blood amino acid level by 30% as much as 40% of the total myocardial oxygen consumption could be accounted for by aerobic metabolism of amino acids. In short, the ability of the heart to utilize various substrates illustrates the great versatility of the myocardium in the source of its fuel supply and has been regarded as an "important factor of safety"[172] in the fuel supply of an organ of vital importance for the survival of the organism.

The work of Bing on human cardiac metabolism was soon refined and expanded in many laboratories, especially by Carlsten and his associates in the laboratory of Lars Werko in Sweden. When these investigators studied myocardial extraction of different substrates in fasting, healthy individuals they found that the plasma content of glucose, lactate and saturated, long chain fatty acids decreased significantly during the passage through the heart.[173] They

found a positive correlation between arterial levels of fatty acids and lactate and myocardial extraction of these compounds. There was no change in total amino nitrogen, but release of alanine and uptake of glutamate by the heart *in situ*.

While studies on arterio-venous differences of metabolites allowed identifying qualitatively the source of energy provision for the mammalian heart muscle, no insight could be gained into the mechanism of how the heart selects its fuels. Addressing this question had to await the development of improved methods for perfusion of the isolated heart *in vitro*, and for the development of isotopic tracer techniques to estimate metabolic fluxes.

Shipp, Opie and Challoner were the first investigators to utilize radioisotopic labeling to track the metabolic fate of glucose and free fatty acids (FFA) in isolated, buffer perfused rat hearts.[174] While this preparation remains a fundamental tool to investigate in depth biochemical/molecular mechanisms regulating myocardial metabolism, it is also notoriously non-physiological for a number of evident reasons. Ideally, energy substrate uptake, oxidation and storage should be directly measured in intact hearts, in situ. However this approach is challenging. Before the study by Shipp and colleagues, several groups, especially C. Lovatt Evans from Starling's laboratory of University College in London, had performed pioneering measurements of substrate metabolism in the intact heart[175-177], although they did not use isotopic tracers and their conclusions about oxidative metabolism were based on assumptions or on calculated respiratory quotient. Lastly, in 1969, Most and collaborators published the first study in which ^{14}C-labeled palmitate infusion and coronary sinus blood sampling were utilized to measure cardiac fatty acid uptake and oxidation, *in situ*.[178] Interestingly, this breakthrough was achieved not in experimental animals, but in human subjects undergoing coronary catheterization. Other groups later refined this method in animal models (and in humans) and

provided fundamental contributions to elucidate the factors regulating fatty acid and carbohydrate metabolism by cardiac muscle.[179] A series of elegant studies in humans by Wisneski and colleagues, during the 1980s, determined that >60% of glucose extracted by the normal heart in the fasting, resting state is probably first stored as glycogen,[180] and defined in great detail the potential limits of isotopic tracer-based measurements of cardiac fatty acid and carbohydrate metabolism.[181] In principle, the methods established then have stood the test of time and are still applicable.[182]

b) Expanded Notes on Coronary Sinus Catheterization and Flow Measurements

Accurate measurements of myocardial blood flow and metabolism require accurate techniques for cannulating the coronary sinus. The coronary sinus in humans and most large animals travels in the left atrioventricular groove and is contiguous with the great cardiac vein. It then receives the posterior, middle, and small cardiac veins prior to emptying into the right atrium (i.e. the majority of venous blood flow with the heart), although it does not receive the anterior cardiac vein draining the free wall of the right ventricle. Thus, flow measurements and blood sampling from the coronary sinus provide the parameters for determining rates of oxygen consumption and substrate utilization by the left ventricle. Of note, the left azygous vein drains into the coronary sinus in pigs and sheep and this anatomical characteristic should be considered to avoid confounding measurements.

Coronary sinus flow measurement using thermodilution was initially described by Ganz et al.[183] The tip of the catheter is usually placed within or just at the junction of the coronary sinus and the great cardiac vein. Saline or 5 percent dextrose at room temperature or colder is infused continuously and retrogradely. The temperature of the blood-saline mixture downstream is monitored by an external thermistor, while the infusate temperature is monitored by an internal

thermistor near the tip of the catheter. The flow computation assumes that heat lost from the system between the site of injection and the detection site is negligible and heat lost by the blood equals the heat gained by the indicator. Coronary sinus catheters also contain a blood sampling port.[184] Dimensions of commercial catheters and distancing of the external thermistors from the tip require a human adult size subject, otherwise the thermistor will sit outside the coronary sinus orifice and the infusate will be diluted by right atrial blood and cause inaccuracies in flow measurement. Furthermore, injection of infusate does tend to dislodge the catheter. In short, the CS thermodilution systems are suitable for certain animals only.

Alternative methods are based on extracorporeal shunts from the coronary sinus to the superior vena cava through flow probes connected in series,[185-187] or on coronary sinus sampling in conjunction with direct measurement of arterial coronary flow. Generally, perivascular ultrasound transit time probes are placed around a coronary artery for flow measurement. These probes can be used acutely in open thorax preparations or implanted for chronic measurements after chest closure. [188-190] A less invasive method that has been used to measure coronary flow in patients, while simultaneously withdrawing coronary sinus blood samples, consists of simultaneously measuring blood velocity with an intravascular Doppler wire and vascular internal cross sectional area with intravascular ultrasound catheter.[191] In any case, sampling coronary sinus blood in conjunction with blood flow measurements in a selected coronary territory does potentially create some errors in the assessment of myocardial oxygen and substrate consumption, because the blood content in the coronary sinus blood reflects global cardiac metabolism.

APPENDIX 13. Expanded Notes on Probing Metabolism in The Isolated Heart *ex vivo*

a) Historical Notes

In the early days of cardiovascular research it was difficult to correlate contractile performance and metabolic activities of the heart. The reasons were as follows. In the blood-perfused heart *in situ* it is impossible, to manipulate the heart's substrate supply with precision. In addition, changes in the work load of the heart are subject to complex hormonal and hemodynamic responses which make it difficult to assess individual parameters known to impact cardiac metabolism. Even in the heart-lung preparation of Starling,[192] which was used extensively in earlier studies, it was not possible to effectively separate metabolism of the heart from that of the lungs.[193] Therefore investigators were unable to assess rates of metabolite disappearance or oxygen consumption in a quantitative way through this earlier method. In this context, Langendorff's retrogradely perfused cat heart preparation was therefore a breakthrough, although it was Langendorff's original intent to demonstrate that the mammalian heart receives its nutrients through the coronary circulation (as opposed to through the endocardium) (1895).[194]

Because from now on investigators viewed the heart as an intact organ with its own vascular supply, several major discoveries during the first half of the last century were made using the Langendorff preparation. These include the oxygen requirement of the heart,[195] the heart's use of glucose,[196] and of fatty acids,[197] as well as the interaction of substrates.[198] Independent from all this, Charles Lindbergh in the 1930s developed an apparatus to circulate liquid under constant pressure in a closed system,[199, 200] which created the foundation for *ex vivo* CO_2 collection and, ultimately, was designed for the preservation of organs for transplantation.

b) Practical Aspects of Isolated Heart Perfusions

The working heart apparatus consists of a cannula assembly for the left atrial and aortic

cannula and five water-graduated chambers kept at 37°C with a recirculating water bath. Following isolation of the heart from the fully anesthetized animal through a wide thoracotomy the beating heart is transferred into a beaker filled with saline and kept in an ice bath. Next both the aorta and left atrium are cannulated, enabling supply of the heart with perfusate at constant left atrial filling pressures and aortic afterloads. Upon rewarming the heart begins to beat. In this set-up, the perfusion buffer, which includes the aortic outflow as well as the coronary effluent, are recirculated into the buffer reservoir. This set-up has been modified to a "semi-recirculating" perfusion system, in which only the aortic outflow (which has not transited through the coronary circulation) is recirculated into the buffer reservoir; the coronary effluent is collected for specific measurements of metabolites released by the heart (for e.g. lactate). A detailed description of the "semi-recirculating" set-up has been described for both the working rat[201] and mouse[202] hearts.

The composition of the perfusate buffer is crucial and a determining factor for any metabolic investigations; this includes considerations about the concentration of ions (for e.g. calcium), but also the choice of substrates, both the type and concentrations. In a typical perfusion experiment, the perfusate buffer usually consists of Krebs-Henseleit solution containing and one or more of the energy-providing substrates. Long-chain fatty acids must be pre-bound to albumin in a specific molar ratio (e.g., 4:1 for palmitate or 6:1 for oleate). A complex mixture of fatty acids or TG may also be considered. It is important that albumin is defatted by charcoal treatment.[203] Lastly, amino acids such as glutamine (or leucine), hormones (for e.g. insulin, epinephrine, etc.), cofactors (for e.g. carnitine) may be added depending on the study's objective, since they will ultimately affect substrate selection for energy metabolism as well as for maintaining contractile function.[19] Irrespective of the substrates, that proper

oxygenation of the system is essential; the addition of fatty acids bound to albumin requires a specific proper oxygenation, which can be achieved by various means.[201-203] In each case, ions, substrates, hormones or cofactors are added at concentrations close to physiological, mimicking the composition of the blood *in situ*, which differs for rats vs. mice. Cardiac function can be assessed by continuous measuring cardiac output, aortic pressure development, heart rate, and left ventricular developed pressures, while oxygen consumption rates can be measured by cannulating the pulmonary artery of the heart, and following AV differences in oxygen content, and coronary flow rates.

The left ventricular ejecting heart preparation has proved very useful in correlating changes in ventricular function with energy metabolic rates. Hearts can be perfused with the various carbon substrates mentioned above, and using isotope labeling techniques to assess metabolic activity (described in the subsequent section), metabolic flux rates can be directly assessed. For tracer studies, considerations should be given, however, to the perfusion set-up (recirculating vs. non-recirculating), because this will impact on data interpretation (described in the subsequent section "Stable isotope labeling by GCMS).

While an advantage of the isolated working rat heart is that external work can be altered (by altering left atrial filling pressures and aortic afterload), the model is basically a left ventricular working preparation, since no right ventricular load is applied. In select studies, investigators have overcome this problem by inserting additional cannulas into the inferior vena cava and pulmonary arteries to produce a bi-ventricular working heart.[204-206] An additional limitation of the isolated working heart is the limited stability of the preparation, especially at higher workloads. However, in the presence of adequate carbon substrates and oxygenation, the isolated working rat heart may be stable for up to 2 hr. Lastly, it must be remembered that

despite providing physiological levels of workload and nutrients, the saline perfused heart is maximally vasodilated[203] and fails to represent the complexity of the *in vivo* situation. This is illustrated by the fact that despite displaying normal values for markers of energy status (for. e.g. phosphorylated level of AMP kinase), energy-related metabolite levels as well as contractile function of hearts from transgenic mouse models (for e.g. PPARα-null or *mdx* mice) are different following perfusion *ex vivo* compared with *in situ*.[19, 207] However, irespective of any limitations, the isolated working heart preparation is the only preparation that can accurately measure cardiac efficiency, as defined by the ratio of energy "out" (cardiac work)/energy "in" (rates of substrate oxidation of O_2 consumption).

APPENDIX 14. Metabolism of Isolated Cardiac Cells and Cell Lines

There are no expanded notes for this section

APPENDIX 15. Expanded Notes on Mitochondria

a) Mitochondria as Regulators of Cell Function and Death

In addition to this unique role as energy transducers through oxidative phosphorylation of ADP, mitochondria also monitor complex information related to the cardiomyocyte's environment and intracellular milieu.[208-210] In addition, mitochondria are implicated in the loss of cardiomyocytes through the release of various factors, including cytochrome c, Smac (second mitochondria-derived activator of caspases), and AIF (apoptosis inducing factor), triggering programmed cell death in various cardiac pathologies.[208, 211] The non-canonical functions of mitochondria, especially the dynamics of mitochondrial fusion and fission, as well as pathways dedicated to surveillance and elimination of dysfunctional mitochondria, are described as an

interaction and are the subject of a recent didactic review[212] and therefore not further discussed here.

b) Isolated Mitochondria

Mitochondrial dysfunction contributes to a broad range of pathologies, particularly those involving the cardiovascular system, with compelling evidence implicating changed bioenergetics in both the vessel wall and the cardiomyocyte. Mitochondria deal with the oxidation of reducing equivalents (NAD-H^+, FADH-H^+) generated by oxidative decarboxylationin the citrate cycle.[12] One of the great advances in bioenergetics is Peter D. Mitchell's chemiosmotic theory of how protons in aqueous solution function in the production of ATP in mitochondria, which earned Mitchell the 1978 Nobel Prize for Chemistry.[213] Other cellular sources of ATP such as glycolysis were understood first, but such processes for direct coupling of enzyme activity to ATP production are not the major source of useful chemical energy in most cells. Chemiosmotic coupling is the major ATP generating process. The principle is present even in chloroplasts and several single celled organisms in addition to mitochondria. Evaluating mitochondrial or bioenergetic function has now moved beyond simply isolating the organelle by disruptive techniques and measuring simple parameters such as respiratory control ratio and State3/4 respiration. The contemporary approach to bioenergetics is guided by the mitochondrial quality control paradigm. This model centers on the dynamic aspects of mitochondrial function and has broadened the biological endpoints relevant to analysis of cellular bioenergetics. The focus in now on how cells under metabolic, genetic or environmental stress maintain the quality of the mitochondrial population. Mitochondrial quality is maintained through a well regulated balance between mitochondrial biogenesis, the maintenance of bioenergetic reserve capacity, mitochondrial fission and fusion, and

mitophagy.[214] Mitophagy is particularly important because it removes damaged mitochondria whose presence may contribute to a progressive and accelerating bioenergetic dysfunction. In the rapidly evolving interface between redox biology, bioenergetics and cardiovascular disease investigators are beginning to question some long held assumptions. For example, it appears that mitochondrial superoxide and hydrogen peroxide production is not a major source of oxidative stress in the cell but an intricate "retrograde" signaling pathway that co-ordinates mitochondrial function with the demands of the cell through regulating transcriptional events in the nucleus. Importantly, new animal models are revealing the impact of mitochondrial genetics in controlling inflammation through retrograde signaling pathways. This is critical in translating the emerging concepts of redox biology and bioenergetics as investigators strive to understand how the pro-oxidant and pro-inflammatory environment associated with the sedentary western lifestyle contributes to increased risk for cardiovascular disease.

c) Translational Bioenergetics

The new, sensitive techniques for the measurement of bioenergetic function (e.g. [ATP], [adenine nucleotides], [phosphocreatine/ATP]) in cells and tissues is lowering the threshold for translating the latest advances in mitochondrial research to the clinic.[215, 216] This includes using the bioenergetics of peripheral leukocytes and platelets as direct or surrogate indices of cardiovascular pathology in patients and animal models.[217] Whether bioenergetic alterations observed in peripheral blood cells reflects similar parameters in the heart still remains to be determined.

APPENDIX 16. Expanded Notes on Proteomics and Posttranslational Modifications

Background to Proteomic Analyses in Metabolic Research

The proteome is complex but, so, too, are single proteins. For example, a protein can have multiple splice variants. More than one amino acid can be modified in a single protein, and a single amino acid residue can be modified by more than one type of PTM. In fact, different PTMs can compete for the same amino acid residue. Within the human cardiac 20S proteasomes, there are 63 Lys that are ubiquitinated lysines and 65 acetylated Lys with about two-thirds (or 39) of them shared.[218] Desmin, an intermediate filament protein involved in the alignment of mitochondria with the nucleus, myofilament and the cell membrane, undergoes GSK 3b-phosphoryation in many models of heart failure, which alter its susceptibility to selective proteolysis and subsequent amyloid formation.[219] The extent of phosphorylation and proteolysis is decreased with resynchronization therapy (CRT) that also shows a reduction of amyloid-like oligomer deposits. The link between phosphorylation and proteolysis in heart failure and their reversal towards control levels with CRT is also observed for the F_1F_0-ATP synthase beta subunit, which produces the majority of the cell's ATP.[220] Further proteomics analysis also revealed that the regulation of this important enzyme is redox sensitive and that the oxidation of a particular Cys residue in the alpha subunit can completely (and dramatically) reduce the enzymatic activity of this important protein complex.[221]

It has been said that proteomics is extending the boundaries of biologic and metabolic research like no other technique before. Proteomics is the qualitative and quantitative study of a large, often cell-wide set of proteins, their isoforms (arising for two or more genes or from splice variants of single gene), and the large number of possible co- and post-translational modifications (PTMs). Proteomics has to take account of: i) the chemical and physical diversity of proteins, ii) the dramatic effects changes in a single amino acid or a co-valent modification or

PTM in a protein can have on one or more of these parameters, and iii) the large dynamic range of protein concentrations within any given body fluid, cell, or tissue, which drives the need to enrichment for low abundant proteins in order for them to be observed in a proteomic experiment. Although technically challenging it is this variability in protein size and structure that underlies biology. DNA is methylated and demethylated by enzymes, all miRNAs are produced via highly regulated protein complexes, and metabolites, metabolism and the mitochondria are influenced, regulated or produced via proteins. The means to quantify accurately this variably across the proteome in a large number of samples has been the goal of most proteomic technology development.

Additional aspects must be considered. Proteomics can be viewed as analytical protein biochemistry that is focused on the detailed and large-scale protein characterization and/or quantification. Proteomic methods can be applied equal to mapping PTMs of a single protein, the identification of binding partners or the subunits comprising a protein complex to the analysis of 1000s of proteins or their PTM forms. Regardless of the biological or clinical question, mass spectrometry (MS) based proteomic most often involves sample digestion using trypsin or another enzyme that has high specificity for the proteolysis of a specific amino acid residue(s) within the polypeptide chain. This approach is also referred to as bottom-up or shotgun proteomics.[222] Trypsin is commonly used because it is highly selective for cleaving after Arg and Lys amino acid residues, which are both highly represented in proteins and also because trypsin is an efficient protease with a broad activity spectrum covering a large pH and ionic strength range. This makes it adaptable to most sample preparation conditions. Because the accuracy of the mass spectrometer's measurement is increased by a smaller mass, measurement of a peptide is more accurate than that of an intact protein. Digestion of proteins when used with

the current high-end mass spectrometer essentially allows for the unambiguously identification of the amino acid sequence of any observable peptide, thereby driving the definitive identification of the protein.

There are currently three major MS-based approaches: *de novo* discovery, targeted, and SWATH (sequential window acquisition of all theoretical fragment-ion spectra). Any technology that characterizes large numbers of proteins falls under the umbrella of proteomic technology. These include gel-based methods (e.g. 1 and 2DE gel electrophoresis), flow cytometry (and the MS equivalent, CyTOF), as well as protein (e.g. antibody ELISA) and aptimer arrays. *De novo* discovery aims to identify as many peptides or their modified forms as possible, whether the sample is a single protein, a subproteome or a whole cell lysate. In *de novo* discovery MS-based experiments, the extent of proteome coverage (the number of peptides observed, the coverage of each protein and/or the number of proteins observed) is the key. Maximizing proteome coverage is determined based on the extent and type of sample preparation, peptide fractionation and the MS instrument, itself. In this approach, the mass spectrometers are used in Data Dependent Acquisition (DDA) in which the instrument selects the most abundant (intense) peptides at any given time and fragment them in order to determine their underlying amino acid sequence (MS/MS). When carrying out MS/MS the instrument misses peptides that were not selected and thus, often the sample is run multiple times to reduce the amount of "missing" data which can be problematic when trying to quantify low abundant proteins or PTMs. Yet, this remains the best approach to finding new discoveries including new proteins[223] and new sites of PTMs. Often the goal of *de novo* discovery is to detect differences between two or more experimental groups. Many papers in the cardiology literature have used *de novo* discovery and have found that many proteins involved in metabolism are altered. It is worthwhile to note that these same MS

instruments can carry out intact mass analysis. This means analysis and quantification of a protein without digestion (sometimes referred to as top down)[224] or partially digested into larger fragments (also referred to as middle down). This method is particularly useful when tracking a protein's PTM status.

Targeted protein quantification using Selective Reaction Monitoring (SRM) and its specialized approaches (Multiple Reaction Monitoring (MRM) and Parallel Reaction Monitoring (PRM)) allow for the quantification of specific targeted proteins and can provide the equivalent data to an ELISA assay. In this approach, peptides comprising an amino acid sequence unique to the target protein are selected within the MS instrument (all other peptides in the sample are essentially excluded from reaching the MS detector). The selected peptide is energetically fragmented and the resulting transitions, representing a unique fragment of the peptide, ensure the correct peptide. Quantification (peak area) of the correct peptide can be easily obtained, either fold change between different samples or the absolute quantity. The latter requires that a known concentration of an isotopically labeled (e.g. ^{15}N) synthetic peptide composed of the same amino acid sequence as the target peptide is added to the sample. Based on a standard curve generated using the ^{15}N labeled peptide, the exact concentration of the endogenous light labeled (e.g. ^{13}N) endogenous peptide can be determined and, hence, the exact concentration of the parent protein. As this method is peptide dependent, one can target peptides which are isoform specific (e.g. TGF beta isoforms 1, 2 or 3)[225] or specific PTMs (e.g. cardiac TnI phosphorylation).[226] Further examples are given below.

SWATH is a newer approach, also known as Data Independent Acquisition (DIA). Although first performed in 2004[227] and later with the development of an MS library for enhanced analysis,[228] SWATH has taken off with the development of new faster mass

spectrometers and the reduced cost for computer memory and computation. SWATH, which is a hybrid between *de novo* discovery and SRM assays, uses the approach in which thousands of known (previously characterized during building of the MS peptide library) proteins are identified and quantified in every sample with every single run without extensive fractionation. This time- and cost-effective technology allows one to carry out extensive, reproducible assessment of the broad proteome on extremely large numbers of samples in a cost effective manner. However, as mentioned above, the MS library only consists of those peptides within the dynamic range of the MS instrument and which have been already preselected. Thus, new discoveries outside of this preselected set of peptides/proteins are not as feasible as it is with *de novo* discovery.

Lastly, large-scale sample preparation remains a limitation to the translation to large sample analysis. There is a need to develop hands-free robotics or automation stations for MS sample preparation; a reasonable goal would be to be able to process 200-500 samples per day with exceptional precision (CV%<15%). The addition of automation that allows for the enrichment of single proteins or specific PTMs (e.g. phosphorylation, acetylation) or target peptides using anti-peptide antibodies would enhance the ability to accurately quantify proteins and their modified forms on a scale not previously possible and move to the need for small sample quantities.

APPENDIX 17. Expanded Notes on Metabolomics

Expanded Notes on Biomarker Studies

Metabolomics attempts to provide a comprehensive identification and quantification of all or select groups of endogenous small molecule metabolites (< 1,500 Da) in a biological

system (heart or plasma) using a high throughput system. Although several technical challenges remain, especially in broad spectrum metabolomics[229] recent methodological advances have improved coverage and identification of metabolites. Because metabolomic studies are at risk for numerous confounders and because of the unpredictability of the onset of pathological states, controlled human studies offer advantages for metabolite biomarker discovery. Clinical cardiology is well suited for such investigation, including experiments in which serial sampling of blood using uniform protocol subjects, before and after a controlled perturbation, allows each individual to serve as his or her own biological control. In addition to attenuating noise attributable to inter-individual variability, such studies allow more precise assessment of the kinetics, and even tissue specificity, of metabolite changes. This strategy has been employed to study the metabolic consequences of effects of exercise, myocardial ischemia, as well as overt myocardial injury.[230, 231] However, it has not yet been established whether changes of the metabolite profile in the peripheral circulation reflect changes in cardiac metabolism *per se*.

APPENDIX 18. Genetic Models for Cardiac Metabolism

Tables A3 and **A4** list metabolically relevant genes and their modifications in the heart with their phenotypes.

APPENDIX 19. Non-genetic Models for Cardiac Metabolism

Table A5 lists non-genetic models with their modifications and cardiac phenotypes.

APPENDIX 20. Expanded Notes on Metabolism of the Developing, Newborn, and Neonatal Heart

a) Comparisons of Cardiac Metabolism in Immature Rodents vs Large Mammals

A dramatic shift in energy substrate utilization occurs immediately after birth. After a brief period of starvation the heart switches from the predominant oxidation of carbohydrates to the oxidation of fatty acids. Although this tenet has been supported by large animal studies performed *in situ*, the mechanisms behind this transition remain debatable. Rodent studies suggest that an increase in PPARα mRNA expression immediately after birth signals this transition.[232] PPARα presumably increases expression and thus activity for carnitine palmitoyl transferase I, a pivotal enzyme regulating long chain fatty acid oxidation. However, no newborn rodent studies to date have actually measured substrate flux in order to confirm the timing of the substrate shift. Several investigations have established the time course for maturation of cardiac energy metabolism in the sheep model *in situ.* Bartelds, *et al* showed that glucose and lactate were the prime energy substrates during ovine fetal life, and the switch to fatty acids as prime oxidative substrate occurred within 2-16 days after birth.[233, 234] Regulation of myocardial oxidative phosphorylation matures within the same age period in parallel with accumulation of the adenine nucleotide translocator protein.[235] Based on rodent data, nuclear receptors such as PPARα operating in conjunction with their co-activators offer a potential mechanism for rapidly integrating these processes shortly after birth. However, studies in sheep show that no increase in PPARα protein content occurs.[236] PGC-1α protein, a coactivator for multiple nuclear receptors increases during this transition in both rodent and sheep hearts, suggesting a potential regulatory signal for the transition. [236]

Fatty acid and carbohydrate metabolism generally exhibit reciprocal regulatory patterns, where one decreases as the other increases.[17] Studies from tissues extracted from newborn sheep heart showed that postnatal increases in CPTI activity and protein content occur, but are

considerably lower than anticipated from the large increase in the rate of LC-FA oxidation observed *in situ* in the same animals. [233] As circulating levels of free fatty acids rise substantially (~10-fold) immediately after birth (and are sustained), substrate supply appears to be a major determinant for the increased preference for LC-FA oxidation around birth. Furthermore, a steady decline in liver (L)-CPTI, the predominant fetal isoform in sheep fetal heart, occurs immediately after birth with maintained M-CPTI protein levels. Thus, CPTI expression does not appear to contribute significantly to the postnatal metabolic transition in sheep. A systemic signaling event which modifies substrate supply appears to play a role. Thyroidectomy does remove many of these signals including the increase in PGC-1α, implying an important role for thyroid hormone in this metabolic transition.[236]

b) Historical Notes on <u>Cardiac Metabolism in the Newborn Period</u>

Size is the principal limitation for assessing newborn metabolism *in situ* and *ex vivo.* Thus, very little is known concerning myocardial metabolism in the human newborn and infant other than conjecture and extrapolation from other species. Furthermore, prior to the 1980s most information defining newborn cardiac metabolism arose from studies performed on isolated cardiac myocytes and/or mitochondria harvested from animal models. Multiple studies were performed on energy-rich phosphates in papillary muscles isolated from newborn rabbit hearts.[237] The developmental metabolic response to ischemia and hypoxia was a principal focus. Those studies in isolated papillary muscle as well as in a few retrograde perfused rat[238] hearts suggested that the newborn heart showed superior ATP synthesis during oxygen deprivation. These presumptions were, however, based on static measures of ATP. This beneficial response presumably occurred due to increase anaerobic glycolytic capacity. However, these preparations were substrate limited as fatty acids were not included in the perfusion medium, and subsequent

studies performed in sheep in situ challenged these concepts.[239, 240] The studies performed in lambs in situ used ^{31}P NMR to measure high energy phosphate kinetics dynamically and showed similar mitochondrial dysfunction between newborn and immature hearts in response to hypoxia.[239, 240] These studies suggested that modeling the newborn or perinatal heart by isolated perfused tissue or heart studies should closely emulate substrate conditions *in situ*, and apply dynamic measurements of flux.

APPENDIX 21. Expanded Notes on Ischemia, Reperfusion, Heart Failure, and Metabolic Modulation

a) Energy Metabolism in the Ischemic, Ischemic-Reperfused, and Failing Heart

This section integrates the application of the different methods used to assess cardiac metabolism. Ischemic heart disease (including acute myocardial infarctions and angina pectoris) profoundly affects cardiac energy substrate metabolism. Alterations in the availability of oxygen and/or a mismatch between oxygen supply and oxygen demand to the heart contributes to the metabolic phenotype(s) during both ischemia and reperfusion. Ischemia, therefore, imposes a major stress on the myocardium and elicits a dramatic perturbation in energy substrate metabolism. Substrate metabolism and contractile function of the heart are inextricably linked. The prerequisite for oxygen in the process of oxidative phosphorylation, described in the main text, results in a rapid decline in ATP production from mitochondrial oxidative phosphorylation that is proportional to the degree of ischemia. This results in a rapid loss of contractile force, a depletion of high energy phosphates, and disturbances of ionic homeostasis.

During **ischemia** glycolysis becomes an important source of energy due to its ability to generate ATP in the absence of O_2. There are several grades of ischemia. In mild to moderate ischemic hearts glucose uptake and the mobilization of endogenous glucose from stored glycogen contribute to increased flux through the glycolytic pathway. Although glycolytic ATP production (assessed by ^{3}H-isotopic methods, or by lactate and alanine production) may be sufficient to maintain/correct ionic homeostasis during mild to moderate ischemia, the hydrolysis of glycolytically derived ATP uncoupled from subsequent pyruvate oxidation leads to the increased generation of lactate and H^+. During severe/total ischemia, the metabolic products of glycolysis accumulate, and flux through the pathway is eventually inhibited due to the inhibition of glyceraldehyde 3-phosphate dehydrogenase by lactate (Lundsgaard effect) or the accumulation of H^+ (intracellular acidosis). These effects can further aggravate disturbances in ionic homeostasis. As glycolysis only provides a small amount of ATP compared to that provided by mitochondrial oxidative phosphorylation, the ability of glycolysis to maintain ionic homeostasis during ischemia is finite. This leads to a sequel of events, including intracellular acidosis, Na^+ overload and Ca^{2+} overload. Intracellular acidosis also impairs myofilament responsiveness to Ca^{2+}, thereby contributing to the loss of contractile force during ischemia, and can contribute to impaired recovery of post-ischemic mechanical function. Another source of anaerobic ATP production is the conversion of α-ketoglutarate to succinate and substrate level phosphorylation of GDP to GTP[241] Succinate also controls reperfusion injury through mitochondrial ROS production.[242]

If reversibly injured ischemic myocardium is reperfused (such as occurs during thrombolytic therapy or percutaneous coronary interventions), rates of mitochondrial oxidative metabolism increase as oxygen supply to the heart is restored. However, while rates of fatty

acid oxidation rapidly recover, the rates of glucose oxidation remain depressed, leading to a decrease in cardiac efficiency and function. The rapid recovery of fatty acid oxidation, via mechanisms operative in the Randle glucose-fatty acid cycle contribute to an uncoupling of the rates of glycolysis and glucose oxidation which persists into the reperfusion period despite the restoration of coronary flow and hence O_2 delivery. This uncoupling of glucose metabolism adds a proton load on the heart during reperfusion, contributing to potential ionic imbalances into reperfusion. These disturbances in ionic homeostasis during ischemia and in the post-ischemic period during reperfusion contribute to deficits in both cardiac function and cardiac efficiency, both of which can be improved by therapies that optimize myocardial energy substrate metabolism.

Alterations in energy substrate metabolism that occur in **heart failure** are complex, in part due to the heterogeneous nature of heart failure itself. Heart failure can occur from a number of cardiovascular conditions, with the more common ones including ischemic heart disease, hypertension, and cardiomyopathies of genetic origin. The metabolic phenotype of the failing heart appears to be at least in part dependent on the stage/severity of the syndrome. As heart failure itself progresses to its advanced stages, mitochondrial function and metabolism is compromised, resulting in a decrease in myocardial ATP and PCr content. Mitochondrial defects in the rates of oxygen consumption and electron transport chain activity negatively impact oxidative phosphorylation and ATP generation. These deficits in myocardial ATP production support the concept that alterations in energy substrate metabolism are important biochemical hallmarks of, and contributors to, the pathogenesis and progression of heart failure. Enhanced regional myocardial glucose uptake, assessed with [18F] 2-deoxy,2-fluoro-glucose

(FDG) and positron emission tomography (PET) is a hallmark of reversibly ischemic, viable myocardium.

As mitochondrial oxidative capacity decrease in the failing heart, glucose uptake and glycolysis increase in an attempt to compensate for the loss of mitochondrial oxidative metabolism (similar to what occurs in the ischemic heart). This phenotypic switch from mitochondrial oxidative metabolism to glycolysis results in the hypertrophied and failing heart reverting to a more "fetal" metabolic phenotype. Accompanying this metabolic switch, is a down-regulation of many metabolic enzymes involved in fatty acid oxidation and an up-regulation of enzymes involved in glucose uptake and glycolysis.

There is conflicting evidence as to the relative source of fatty acid oxidation versus glucose metabolism in the failing heart. In hearts subjected to pressure overload with preserved ejection fraction, the rates of fatty acid β-oxidation are similar relative to normal hearts, whereas the rates of glycolysis are accelerated. Furthermore, fatty acid β-oxidation rates also similar in hearts subjected to a myocardial infarction and subsequent heart failure at a time point when there is a down regulation in genes encoding enzymes involved in fatty acid oxidation. Fatty acid oxidation also does not differ in acute heart failure secondary to aortic banding in rats. However, in dogs with pacing-induced dilated cardiomyopathy, and in severe heart failure, fatty acid oxidation is reduced, while glucose oxidation is increased,[190] where glucose uptake and oxidation are also preserved relative to the normal heart. Patients with asymptomatic hypertrophic cardiomyopathy and NYHA Class II and III heart failure also have preserved cardiac fatty acid oxidation, but patients with dilated cardiomyopathy display reduced fatty acid utilization/oxidation. This is well documented.[191, 243]

While glycolysis rates are elevated in the failing hearts, glucose oxidation rates can be depressed. This may occur due to the depressed mitochondrial oxidative metabolism, as well as the result of an inhibition of glucose oxidation due to the preserved fatty acid oxidation in hearts with compromised mitochondrial oxidative metabolism. The decrease in glucose oxidation results in an increased uncoupling of glycolysis from glucose oxidation in the hypertrophic and failing heart, that can lead to an increase lactate and H^+ production, and a decrease in cardiac efficiency, and may thus represent a viable therapeutic target to improve cardiac efficiency by modulating the balance between fatty acid and glucose utilization. This applies, in particular, to clinical and experimental models of both, lipotoxicity and glucotoxicity.

b) Pharmacological Agents Affecting Cardiac Metabolism

STZ inhibits the enzyme O-GlcNAcase in pancreatic β-cells and destroys them. In rodents a high fat diet in combination with a single intraperitoneal injection of *STZ* (15-25 mg/kg) induces cardiac metabolic changes that mirror the decrease in glucose metabolism and increase fat metabolism akin to a type 2 diabetic phenotype,[244] whereas higher doses of STZ (30-55 mg/kg) more closely recapitulate type 1 diabetes.[245-247] Before administration, STZ needs to be dissolved in either glycine or citrate, and a control group consisting of STZ plus insulin treatment is required to exclude any off-target effects of STZ. STZ is toxic and can be potentially harmful if handled incorrectly. Methylglyoxal is a toxic by-product of glycolysis.[248] Chronic administration of methylglyoxal causes cardiac changes akin to diabetes in rat heart.[249]

The PPARα agonist WY-14643, when given for 8 days to rats subjected to left ventricular pressure overload by ascending aortic banding, causes severe contractile dysfunction of the hypertrophied heart,[250] while chronic treatment of rats given WY-14643 (25 mg/kg/d) given by oral gavage for 14 days impairs contractile function of normal hearts in parallel with

decreased mitochondrial respiratory function and increased uncoupling.[251] These are just examples for the strategic use of pharmacologic agents in the assessment of cardiac metabolism. Other examples include drugs targeted at specific enzymes or metabolic pathways such as trimetazidine,[252, 253] ranolazine,[254, 255] resveratrol,[256] rapamycin,[102, 257] metformin,[258] oxygen derived free radical scavengers,[259] and 6-aminonicotinamide.[260] The list is far from complete. This Appendix section complemented by the section on Metabolic Modulators, which includes a more detailed discussion on metabolic adaptations and maladaptations of the heart to hemodynamic, neurohumoral, ischemic and metabolic stress,[261] and offer a particular perspective on metabolic aspects of myocardial ischemia, reperfusion ischemic preconditioning and cardioprotection, hypertrophy and heart failure.[262, 263]

APPENDIX 22. Expanded Notes on Epidemiologic, Genomic, Plasma Proteomic, and Metabolomic Approaches

a) Historical Notes

Large scale approaches to human gene discovery and metabolic profiling form part of population research in cardiovascular disease (CVD), whose origins date back about 65 years. At that time, the Framingham Heart Study launched the recruitment of an unselected cohort from its namesake community to undergo clinical evaluation and long-term follow up with the aim of identifying factors predisposing to coronary heart disease (CHD). The success of this paradigm for determining key environmental, behavioral and biological "risk factors" for CVD in this middle-aged community of European descent spawned a number of population-based cohorts targeting different age, race-ethnic, or national groups in the U.S. and abroad that have incorporated progressively more advanced biochemical and imaging techniques in their

evaluations.[264] Such expansion of population-based research has provided new insights into CVD earlier and later in the life course, and illuminated the nature of race-ethnic disparities and their determinants. Yet the advent of the "-omics" revolution has opened the vast scale of the human genome and its biochemical products for investigation, rendering the thousands of participants included in individual cohort studies suddenly insufficient for the study of a myriad candidate genetic and molecular variants.[264, 265] This has fostered the assembly of consortia involving tens and even hundreds of thousands of participants across cohort studies. More generally, it has brought into focus the need for more efficient, lower-cost approaches to evaluation and follow-up of large populations by leveraging modern technologies and electronic health records.[264] As community-based studies are prompted to innovate by budget constraints, technological advances, and the need for larger populations, more efficient approaches to data collection will characterize the population science of the future.

b) Expanded Notes on Epidemiologic Approaches

The randomized trial sits atop the hierarchy of study designs in human populations, testing the efficacy of interventions in an experimentally controlled manner and thereby allowing direct investigation of cause and effect.[264] The approach has inherent drawbacks, however, including a necessarily narrow scientific focus, and ethical barriers to the study of harmful exposures. The optimal design for research discovery is the prospective cohort study, which affords the opportunity to evaluate associations between a range of exposure variables measurable at study onset, and future development of outcomes of interest. The validity of such associations rests with minimizing bias in the study design with respect to selection of participants and ascertainment of information. It also depends on appropriate control for confounding factors, and upon limiting the play of chance. The temporality of such longitudinal

studies lends support to a potentially causal relationship, but because of unmeasured confounding, this observational design cannot determine whether the association is causal.[266] It is by testing of suitable interventions to modify the risk factors so identified through randomized trials that the association can be deemed to be causal. Causal inference is also possible by harnessing random assortment of genetic determinants of risk factor levels–an approach known as Mendelian randomization.[266]

Studies of "extreme phenoytpes" have also helped to elucidate new cardiometabolic biomarkers in humans. In an initial proof of principle study, Newgard and colleagues profiled obese versus lean humans to gain a broad understanding of the metabolic and physiologic differences in these two disparate groups. Their studies identified a branched-chain amino acid signature that was highly correlated with metrics of insulin resistance.[267] Studies to elucidate novel disease predictors in large epidemiological cohorts are now emerging as well, in some cases complementing prior investigations. For example, a signature of branched chain and aromatic amino acids was also found to have a significant association with future type 2 diabetes, up to 12 years before the onset of overt disease.[268] Similar findings were also obtained in a much larger population of Finnish men.[269] With regards to coronary heart disease, a biomarker composed of dicarboxylacylcarnitines is associated with predictive power of death and myocardial infarction in both derivation and replication cohorts.[270, 271]

c) Genomics and Cardiovascular Risk Assessment

Although the familial clustering of coronary heart disease (CHD) has been appreciated for at least half a century, the identification of specific genetic determinants contributing to this heredity is a more recent development. The advent of genome-wide arrays has enabled genome-wide association (GWA) studies relating common variants (minor allele frequency > 5%) with

the risk of CVD. To date, GWA studies have identified nearly 50 common variants related to CHD.[272] Approximately 20% of the known CHD variants are related to hyperlipidemia. Thus, the majority of identified variants have no apparent relation to any conventional CVD risk factor, high-lighting the persistent gaps in our understanding of pathways leading to myocardial infarction or CHD.

For common variants, the contribution of any individual SNP to overall cardiovascular risk is small. For instance, the per allele excess risk for known CHD variants ranges from 5% to 30%.[272, 273] The strongest associations exist for variants at the 9p21 locus; individuals homozygous for the minor allele have 30-60% increased risk of myocardial infarction compared with major allele carriers.[274, 275] The 9p21 locus does not contain any known genes. It has been proposed that enhancer elements at the locus mediate inflammatory responses, but the true underlying mechanisms remain undefined.

Given the small contribution of common polymorphisms to overall coronary risk, it is not surprising that they add only modestly to the ability to assess risk in a given individual. For instance, Ripatti *et al*. reported that genotypes at 13 loci associated with CHD did not significantly raise the area under the receiver-operating characteristic curve (AUC, a measure of discrimination) or net reclassification index (a measure of risk categorization), compared with traditional risk factors and family history.[276]

A significant proportion of the unexplained heritability in CVD could arise from uncommon (1-5% minor allele frequency), rare (<1%), or private genetic variants. Indeed, rare mutations can have a strong influence on CVD risk, as evidenced by the LDL-receptor variants that underlie familial hypercholesterolemia. Though rare or private mutations can provide insight regarding disease pathogenesis, they do not currently contribute to risk assessment in a

meaningful manner. Genotyping for coding genetic variants with higher allele frequencies (1-5%) has become possible with "exome chips," and clinical studies are beginning to explore the utility of these genotyping platforms.

d) Metabolomics and Cardiovascular Risk Assessment

The early application of "-omics" for biomarker discovery in epidemiologic cohorts has largely involved metabolomics, rather than proteomics. One advantage is the much lower number of circulating small molecules compared with circulating proteins.[265] Several early studies have used mass spectrometry-based approaches. For instance, Framingham investigators identified 5 branched chain and aromatic amino acids that predicted diabetes up to 12 years prior to the onset of disease.[268] A score comprising the 3 amino acids with the strongest association identified individuals with a 5-fold excess risk of diabetes. Shah *et al*. identified a set of branched-chain amino acid and urea cycle metabolites that predicted CVD.[271] Incorporation of these metabolites into risk models led to small improvements in discrimination (AUC).

In a study of patients undergoing elective cardiac catheterization, Wang *et al*. identified 3 metabolites in the phosphatidylcholine-choline metabolic pathway that were associated with CVD.[277] Tang *et al*. showed that plasma concentrations of one of these metabolites, trimethylamine-*N*-oxide (TMAO), were associated with future cardiovascular events in a cardiac catheterization cohort.[278]

These studies demonstrate the feasibility of leveraging "-omics" technologies for biomarker discovery in humans. A large number of additional disease biomarkers are likely to be identified in the coming years, as datasets grow and technological advances lead to improved breadth and sensitivity. Testing these biomarkers in implementation trials will ultimately be needed to advance these discoveries to clinical practice.

APPENDIX Metabolite Concentrations and Flux

Table A1 Concentrations of Major Metabolites in Rat Heart

Tissue or mitochondrial concentrations

a) **Units**: ml/g dry wt	
Tissue water	3.76
Intracellular water	2.00
Mitochondrial matrix water	0.15
b) **Units**: mg/g dry wt	
Mitochondrial protein	400-500

Tissue or mitochondrial concentrations

Units: μmol/g dry wt	
ATP (whole tissue)	25
ADP (whole tissue)	4
AMP (whole tissue)	0.5
P_i	8.5
Mitochondrial (ATP + ADP)	3.2 – 4.2
(NAD^+ + NADH) whole tissue	4.25
(NAD^+ + NADH) mitochondria	3.1
(CoA + acetyl-CoA) whole tissue	0.38
(CoA + acetyl-CoA) mitochondria	0.4 – 0.5
Long-chain acyl-CoA (whole tissue)	0.05 – 0.15

Whole-tissue concentrations

Units: μmol/g dry wt	
Glycogen (glycosyl units)	85
Uridine diphosphate glucose	1
Glucose 1-phosphate	0.05
Glucose 6-phosphate	1.3
Fructose 6-phosphate	0.32
Fructose 1, 6-diphosphate	0.18

Glycerol phosphate	0.35
Dihydroxyacetone phosphate	0.03
Glyceraldehyde 3-phosphate	0.12
3-Phosphoglycerate	0.12
2-Phosphoglycerate	0.014
Phosphoenolpyruvate	0.019
Pyruvate	0.07
Lactate	2.8
Alanine	5.2
Aspartate	11.8
Glutamate	18
Acetyl-CoA	0.01 – 0.03
CoA	0.35
Acetylcarnitine	0.4
Carnitine	2.8
Citrate	0.7
Isocitrate	0.09
2-Oxoglutarate	0.4
Succinyl-CoA	0.2
Succinate	1
Fumarate	1
Malate	0.5
Oxaloacetate	0.07
Free fatty acids	0.21
Triacylglycerol	12.6
Diacylglycerol	0.1
Monoacylglycerol	0.6
Phospholipid	171

Values for Langendorff perfusion at 60mm Hg perfusion pressure, with 5.5 mM glucose and 2 to 200 mU/ml insulin in the perfusate. Hearts were obtained from fed, normal rats, perfused and freeze-clamped (while beating) with aluminum tongs at the temperature of liquid N_2.

Source: Randle, PJ and Tubbs PK. Carbohydrate and Fatty Acid Metabolism. Handbook of Physiology, 1979~~7~~[4] and updated by the authors of the statement.

Table A2: Examples of Pre-Clinical and Human Applications of Radionuclide Metabolic Imaging

Disease Process	Pre-Clinical Imaging	Human Imaging
Atherosclerosis		
Ischemic Cardiomyopathy	Demonstrated PET detection of preservation of glucose metabolism differentiated ischemic but viable myocardium from scar	FDG-PET is now the clinical gold standard for detection of viable myocardium
Myocardial Ischemia	PET using ^{18}F-FBEM-Cys^{40}-exendin-4 revealed a dynamic pattern of prolonged GLP-1 upregulation in the infarcted/ischemic area after ischemia/reperfusion which may be cardioprotective due to stimulation of glucose uptake.[279]	FDG-PET demonstrated enhanced myocardial glucose uptake with exercise-induced ischemia that persisted for 24hr, termed "ischemic memory".[280]
Inflammation	Feasibility of measuring myocardial inflammation FDG-PET demonstrated in a post-MI mouse model with FDG-uptake mirroring the influx of innate immune cells.[281]	FDG-PET is now increasingly being used detect cardiac sarcoidosis and prosthetic valve and device infection
Myocardial Hypertrophy	FDG-PET of rodent heart has confirmed the up-regulation of glucose metabolism tracks directly with increasing hypertrophy and may be a prerequisite for the functional and structural consequences of hypertrophy.[282]	Human PET studies using 1-^{11}C-palmitate has demonstrated the reduction in myocardial fatty acid oxidation is associated with a decline in left ventricular efficiency and an independent predictor of left ventricular mass in hypertension and parallels the development of left ventricular hypertrophy in patients with hypertrophic cardiomyopathy due to polymorphisms in the α-tropomyosin gene.[283, 284]
Cardiometabolic Disease	PET with various metabolic radiotracers in both obese and non-obese models of type-2 diabetes mellitus demonstrated a decline in myocardial glucose uptake and an increase in fatty acid uptake and oxidation. The specific abnormalities in fatty acid metabolism are obesity related and the decline in glucose uptake was paralleled by a decline in GLUT-4 gene expression and insulin-mediated phosphorylation of Akt.[285, 286]	Numerous PET studies using a variety of radiotracers have demonstrated a progressive increase in myocardial fatty acid metabolism and decrease in glucose uptake as one progresses from lean to obesity alone to obesity with type-2 diabetes mellitus with the severity of the abnormalities being related to the magnitude of systemic insulin resistance. There appears to be a sexual dimorphism in the metabolic

		remodeling (women have a greater increase in fatty acid metabolism and men a greater decline in glucose uptake) that also impacts the myocardial metabolic and functional response to various anti-diabetic therapies.[287, 288]

Table A3: Genetic Models of Major Steps in Metabolic Pathways

Gene	Modification	Comments
GLUT1	1- Constitutive overexpression in cardiomyocytes	1- Increased basal glucose uptake. Protected against pressure overload LVH dysfunction, age-related decline in cardiac function, rescued PPARα deficiency but exacerbated LV dysfunction on a high-fat diet.[289-292]
	2- Inducible overexpression in cardiomyocytes	2- Increased basal glucose uptake. Does not protect against pressure overload LVH dysfunction, but increases HIF1 signaling and reduces fibrosis.[293]
	3- Constitutive cardiomyocyte KO	3- Reduced glycolytic induction following TAC, but FAO increased. No accelerated transition to heart failure.[294]
GLUT4	1- Germline KO	1- Massive cardiac hypertrophy decreased circulating FFA. Increased myocardial glucose uptake.[295, 296]
	2- Constitutive cardiomyocyte KO	2- Less cardiac hypertrophy than germline KO, preserved baseline contractile function, increased GLUT1and basal glucose uptake, reduced insulin mediated glucose uptake, decreased recovery from ischemia, cytosolic oxidative stress causes hypertrophy.[297-299]
Hexokinase II	1- Constitutive overexpression	1- Increased glycolysis, improved function in diabetes model, resists ischemic dysfunction, increased PPP flux, reduced oxidative stress and hypertrophy following TAC.[300-303]
	2- Heterozygous KO	2- Increased dysfunction following ischemia, increased hypertrophy following TAC, loss of ischemic preconditioning.[304-306]
PFK	Constitutive overexpression of 6-phosphofructo-2 kinase/fructose-2,6-bisphosphatase	Decreased glycolysis, LV dysfunction and cardiac hypertrophy.[307]
OGT	1- Constitutive cardiomyocyte KO	1- Embryonic lethal.[308]
	2- Inducible cardiomyocyte KO	2- Exacerbates heart failure when expression is deleted after TAC.[309]
LpL	1- Constitutive overexpression of membrane anchored LpL	1- Heart failure secondary to lipotoxicity, which can be partially reversed by limiting de novo ceramide synthesis or by increasing lipid excretion following overexpression of ApoB.[310-312]
	2- Constitutive cardiomyocyte KO	2- Animals develop hypertriglyceridemia, increased basal cardiac glucose uptake and glucose oxidation. FA oxidation is decreased as is TAG droplet synthesis. Normal basal function in young mice, but accelerated HF following TAC, AngII or DOCA induced hypertension. LpL generates PPARα ligand. Increasing glucose availability by crossing with GLUT1 TG mice rescues LV dysfunction.[313-318]
	3- Inducible KO in the heart	3- Animals develop hypertriglyceridemia but develop acute LV dysfunction in following loss of LpL in the adult heart.[319]
CD36	1- Germline KO	1- Reduced myocardial FA uptake and oxidation, increased glucose oxidation. Hearts are protected against high fat diet or age –related cardiac dysfunction. Improved recovery from I/R injury. Prevents lipotoxicity in PPARα transgenic mice. A role in mitochondrial versus sarcolemmal FA uptake is controversial.[320-325]

	2- Constitutive KO in cardiomyocytes 3- Adenoviral overexpression in rat hearts 4- Transgenic overexpression in SHR rats	2- Increased glucose oxidation and decreased FAO. Increased recovery from I/R injury.[326] 3- Increases FA uptake and TAG pool turnover.[167] 4- Increases arrhythmias.[327]
FATP	Constitutive overexpression in cardiomyocytes	Increased FA uptake and oxidation. Abnormal cardiac electrophysiology, diastolic dysfunction.[328]
FABP	Germline KO	40-50% reduction in FA uptake in sarcolemmal vesicles from homozygous KO mice, no change in heterozygous KO mice.[329]
ACSL1	Constitutive overexpression in cardiomyocytes	Lipotoxic cardiomyopathy, that can be rescued by increased hepatic FAO by leptin or alpha-lipoic acid or by increasing cardiomyocyte TAG synthesis with DGAT1 overexpression.[330-333]
CPT1	1- Germline KO 2- Adenoviral Overexpression	1- Heterozygous KO animals develop increased LV dysfunction and lipid accumulation following TAC.[334] 2- Overexpression of L-CPT1 in rat hearts increases glucose oxidation, reduces FAO and mimics the metabolic features of LVH, in the absence of changes in malonyl CoA. Increases markers of pathological LVH.[119]
LCAD	1- Germline KO of LCAD	1- LVH, LV dysfunction, lipid accumulation, increased glucose oxidation, decreased FAO, decreased anaplerosis in fasted animals. Systemic and cardiac changes ameliorated with treatment with LCAD adeno-associated virus.[335-338]
DGAT1	1- Germline KO 2- Constitutive overexpression in cardiomyocytes	1- Cardiac hypertrophy with preserved LV function, increased glucose uptake, decreased PPAR target expression implying decreased FAO.[337] 2- Increased TAG, ceramide and DAG. Cardiac hypertrophy with preserved LV function. Rescues lipotoxic cardiomyopathy in MHC-ACS and MHC-PPARα transgenic mice.[335, 339]
ATGL	1- Germline KO 2- Constitutive cardiomyocyte KO 3- Inducible cardiomyocyte KO 4- Constitutive cardiomyocyte overexpression	1- LVH, dramatic cardiac steatosis, heart failure and premature mortality.[340] 2- LVH, cardiac steatosis and mitochondrial dysfunction. Heart failure rescued by PPAR ligand.[341] 3- Lipid accumulation, increased fibrosis, LV dysfunction, decreased FAO without changes in PPARα target genes.[342] 4- Transgenic overexpression reduces TAG pool, decreases FAO and increases glucose oxidation. Increases the resistance to cardiac dysfunction secondary to diabetes, high fat feeding or pressure overload.[343-346] Molecular mechanisms for substrate metabolic changes remain to be elucidated.
Perilipin	Constitutive cardiomyocyte overexpression	Lipid accumulation and LV dysfunction on the basis of inhibition of ATGL function.[347]
Mitochondrial Complex I	Constitutive cardiomyocyte KO of Ndufs4	Normal basal function, but LV dysfunction under stress. Reduced NAD+/NAD ratio inhibits Sirt3 activity to increase protein acetylation and sensitivity to MPTP opening.[348]
Creatine Kinase	1- Germline KO of GAMT 2- Germline KO of	1- Normal baseline cardiac function. Impaired inotropic responses in vitro, but no aggravation of LV dysfunction in response to coronary ligation and to exercise in vivo.[349, 350] 2- LVH and mild dysfunction on mixed genetic background, but LV

	muscle or mitochondrial CK	dysfunction when backcrossed to the C57BL6 background.[350-352]
	3- Constitutive overexpression of creatine transporter in cardiomyocytes	3- Glycolysis is decreased. Mice develop progressive LVH and LV dysfunction, but have increased recovery from I/R injury.[353-355]

Table A4: Genetic Models of Regulatory Pathways Impacting Myocardial Fuel Metabolism

Gene	Modification	Comments
ACC	1- Germline Deletion	1- Normal LV function decreased LV mass, increased FAO, decreased TAG and Increased Glucose Oxidation.[356]
	2- Constitutive deletion in cardiomyocytes	2- Decreased malonyl CoA, increased FAO. And O_2 consumption. Resist LV dysfunction following TAC while maintaining increased FAO.[334]
	3- Germline knock in of phosphorylation defective ACC	Despite increased malonyl CoA, FAO was unchanged under basal conditions and under conditions of hemodynamic stress that is associated with AMPK activation.[357]
MCD	Germline KO	No difference in FAO in unstressed hearts despite increased malonyl CoA, but increased glucose utilization and improved cardiac efficiency after I/R. Decreased infarct size after coronary ligation in vivo. Increased glucose utilization after HFD. [358-361]
PDHK4	1- Germline KO	1- Increased recovery after ischemia/ reperfusion and reduced infarct size in association with increased glucose oxidation.[361]
	2- Constitutive overexpression in cardiomyocytes	2- Decreased glucose oxidation, increased FAO, no worsening of recovery following I/R injury, defends against diet-induced obesity LV dysfunction, Increased AMPK, increased PGC-1α, and increased mitochondrial biogenesis.[362]
PPARα	1- Germline KO	1- Decreased FAO, preserved LV function in non-stressed hearts but impaired reserve in response to increased workload.[290, 363]
	2- Constitutive cardiomyocyte overexpression	2- Lipotoxic cardiomyopathy that is associated with oxidative stress, increased FAO and increased turnover of TAG pool. Phenotype is attenuated by loss of LpL and by treatment with dietary, medium chain FA.[166, 316, 364-366]
PPARγ	1- Constitutive cardiomyocyte overexpression.	1- Transactivates many genes involved in FAO (overlapping with some PPARα targets); induces lipotoxic cardiomyopathy that can be rescued by PPARα deficiency despite increased expression of PPAR targets that induce FAO and TAG synthesis. However does protect against cytokine-mediated LV dysfunction. Exhibit increased propensity to arrhythmias.[367-370]
	2- Constitutive cardiomyocyte deletion	2- Induces cardiac hypertrophy and augments ANGII-induced cardiac fibrosis.[371]
	3- Inducible cardiomyocyte deletion	3- Reduced expression of CD36, FABP and CPT1. Decreased FAO, LV dysfunction and cardiac hypertrophy.[372]
	4- Constitutive endothelial cell KO	4- Reduces FA uptake into cardiac and skeletal muscle.[373]
PPARδ	1- Constitutive cardiomyocyte deletion.	1- Decreased expression of FAO genes, lipid accumulation, LV dysfunction and heart failure[374, 375]
	2- Inducible cardiomyocyte	2- Decreased PGC-1α, decreased expression of anti-oxidants, decreased glucose and FA oxidation, cardiac hypertrophy and LV

	deletion	dysfunction. [376]
	3- Constitutive cardiomyocyte overexpression	3- Increased glucose utilization. No induction of FAO gene expression. Improved recovery following I/R injury.[377]
	4- Inducible cardiomyocyte overexpression	4- Increased expression of PGC-1α, increased mitochondrial biogenesis, increased expression of FAO and glucose metabolic pathways, increased expression of anti-oxidants, resists pressure overload LVH –mediated dysfunction.[378]
PGC-1α	1- Germline KO	1- Decreased expression of OXPHOS and FAO genes. Decreased FAO, increased glucose oxidation. Accelerated heart failure after TAC.[379-382]
	2- Inducible cardiomyocyte overexpression	2- Massive mitochondrial biogenesis and heart failure.[383, 384]
	3- Human bacterial artificial chromosome (BAC) transgenic	3- Maintaining physiological levels of PGC-1α does not restore LV function after TAC, despite maintaining FAO and preventing induction of glycolysis.[385]
PGC-1β	1- Germline KO	1- Repression of subset of OXPHOS, FAO and Oxidative stress genes. Normal function in non-stressed hearts, but increased risk of heart failure after TAC.[386]
	2- Constitutive cardiomyocyte KO	2- Phenocopies germline KO. However, when combined with PGC-1α deficiency develops perinatal heart failure and perturbed mitochondrial morphology and impaired cardiolipin synthesis.[387-389]
ERR-alpha	Germline KO	Co-activates PPAR targets in the heart and loss of ERR accelerates heart failure in response to pressure overload.[390, 391]
p53	Germline KO	Reduced oxidative stress and ROS generation in models of diabetes.[392]
HIF1-alpha	Constitutive KO of HI1-alpha	Reduces PPAR gamma activation and lipid accumulation in pressure overload cardiac hypertrophy.[393]
Insulin Receptor signaling	1- Constitutive cardiomyocyte KO of IR	1- Increased glycolysis, decreased FAO and glucose oxidation secondary to mitochondrial dysfunction. Increased mitochondrial dysfunction after and accelerated LV dysfunction after coronary ligation. Increased LV dysfunction after TAC or isoproterenol.[394-398]
	2- Constitutive KO of IRS1 or IRS2	2- Decreased hypertrophic response to exercise training. Blunted increase in FAO or glucose oxidation in response to exercise-induced cardiac hypertrophy. Diminished increase in mitochondrial oxidative capacity in response to exercise.[399]
	3- Constitutive KO of IRS1 and IRS2	3- Heart failure secondary to increased autophagy and mitochondrial dysfunction.[400]
	4- Constitutive KO of IGF-1 Receptor	4- Decreased hypertrophic and mitochondrial bioenergetics adaptation to physiological cardiac hypertrophy.[401]
PI3K/Akt	1- Constitutive cardiomyocyte overexpression of activated (CAPI3K) or dominant	1- CAPI3K increases mitochondrial FAO mimicking that of exercise training, whereas DNPI3K blocks the exercise-induced increase in mitochondrial metabolic capacity. PI3K-mediated activation of mitochondrial function following exercise is Akt independent. Insulin mediated GLUT4-mediated glucose uptake is decreased in CAPI3K hearts.[402, 403]

	negative PI3K 2- Constitutive activation of Akt in cardiomyocytes 3- Inducible activation of Akt in cardiomyocytes	 2- Increased basal glucose uptake that is independent of GLUT1 or GLUT4 translocation.[404] 3- Decreased insulin-mediated glucose uptake, despite normal translocation of GLUT4.[402]
mTOR	Inducible cardiomyocyte deletion	Decreased FAO increased glucose oxidation. Normal PGC-1α, but decreased FAO gene expression.[405]
AMPK	1- Constitutive expression of dominant negative AMPK 2- Constitutive cardiomyocyte expression of activating mutant 3- Constitutive cardiomyocyte-restricted KO of LKB1 4- Models of Paracrine regulation of AMPK	1- Decreased induction of GLUT4 translocation and glycolysis following ischemia, and increased injury following ischemia. However during reperfusion, decreased reliance on FAO correlates with increase functional recovery.[406-408] 2- Expression of the mutant of the AMPKγ subunit that is associated with glycogen storage cardiomyopathy revealed metabolic remodeling that drives glycogen synthesis and mechanisms for cardiac hypertrophy that are independent of glycogen synthesis.[409, 410] 3- Decreased AMPK activation and decreased recovery from I/R.[411, 412] Mice harboring mutations of paracrine mediators that increase AMPK activations such as urocortin or macrophage inhibitory factor have increased injury following ischemia.[413-415]
Adiponectin	Global overexpression of Adiponectin receptor 1	Reduces lipid accumulation and oxidative stress following diet-induced obesity.[416]
Sirtuins	1 - Sirt1	Germline Sirt1 heterozygous KO mice and cardiomyocyte-restricted transgenic animals have been used to demonstrate a novel role for Sirt1 acting in concert to repress ERR transactivation of mitochondrial metabolic pathways in pressure overload hypertrophy.[417]
Uncoupling Proteins	Germline KO of UCP3	UCP3 prevents the reduction in cardiac efficiency that occurs with diet-induced obesity, but not in mouse models of leptin deficiency or leptin resistance.[418-420]
miRNA	Cardiomyocyte-restricted KO or overexpression of MED 13	Inhibition of miR208a, induces MED13. Overexpression of MED13 in cardiomyocytes renders mice resistance to the systemic effects of HFD, whereas cardiomyocyte deletion of MED13 increases obesity and insulin resistance.[421]

Table A5 Non-genetic Models of Altered Cardiac Metabolism

Dietary or Non-dietary Interventions	Modifications	Comments
Low Calorie/ Component Deficiency	1 – Caloric restriction a) Fasting	1a) - Short term caloric restriction promotes cardioprotection from ischemia/reperfusion, age-dependent cardiac hypertrophy and diastolic dysfunction[422-425]; these benefits for the heart are mediated by increased activation of AMPK.[422, 425]
	b) Starvation	1b) - Long term caloric restriction, 3-10 month starvation, preserves cardiac contractility and improves cardiomyocyte function and survival via induction of autophagy.[426-429]
	2 – Vitamin Deficiency a) Vitamin D	2a) Vitamin D deficiency causes oxidative stress, apoptosis, and fibrosis in the heart; this leads to left ventricular hypertrophy and lower fractional shortening and ejection fraction. Vitamin D3, specifically, has also been shown to lead to re-endothelialization and vascular repair. [430, 431]
	b) Vitamin E and High Protein (Methionine)	2b) Tocotrienol-rich fraction (TRF) has effects similar to that of folate in reducing high-methionine diet-induced hyperhocysteinemia and oxidative stress in rats' hearts; however, was better than folate at preserving heart glutathione peroxidase enzyme activity.[432]
	3 – Mineral Deficiency a) Selenium	3 – Selenium deficiency mediates cardiac dysfunction via ER stress[433]
	4 – Hypoxia a) Intermittent and Chronic	4a) Hypoxia, both intermittent and chronic, can induce apoptosis and increased expression of NF-kappaβ; hypoxia ultimately leads to RV hypertrophy and failure. This can be attenuated by increased expression of miR-133a.[434-436]
	b) With Copper depletion	4b) Depletion of copper did not have any additional deleterious effects compared to hypoxia alone[435]
Isocaloric and Unbalanced	1 – High Sucrose	1 – Decreased glucose uptake and increased rates of glucose and oleate oxidation. Rates of uptake and oxidation are better matched. Insulin resistance induced by HSD lessens cardiac fuel toxicity.[437]
	2 – High Fructose a) High Fructose alone	2a) – Caused lipid abnormalities in the heart and impaired cardiac insulin signaling; lipoic acid may be useful as cardioprotective agent for the heart in insulin-resistant state. Cardiac insulin resistance manifested by impairment of Akt/endothelial NO synthase (eNOS) signaling was; Akt phosphorylation at Ser(473) and Thr(308), and eNOS at Ser(1177) decreased, while the phosphorylation of eNOS at Thr(495) was increased[438-440]

	b) With pressure overload	2b) - Increased LV mass, decreased ejection fraction; increase in cardiac mass and contractile dysfunction.[441]
	c) With exercise	2c) Swimming exercises improved glucose homeostasis and decreased leptin, resistin, and HOMA-IR index; these changes subsequently led to the amelioration of left ventricular function.[442]
	d) With cholesterol (HCF diet)	2d) – Impairment of contractile function which may lead to cardiomyopathy.[443]
	e) With pharmacologic agent (Lipoic Acid)	2e) Lipoic acid improved insulin sensitivity and enhanced cardiac antioxidant status in fructose-fed rats; implicates the use of lipoic acid as a cardioprotective agent in insulin-resistant states.[438]
	3 – High PUFA (polyunsaturated fatty acids)	
	a) Alone	3a) Supplementation with DHA but not EPA altered mitochondrial phospholipid fatty acid composition and delayed Ca2+ -induced MPTP (mitochondrial permeability transition pore) opening conferring cardioprotection.[444] However, PUFA's have also been shown to increase cardiac susceptibility to lipoperoxidation, leading to increased oxidative stress.[445]
	b) With Pressure overload	3b) Decreased mitochondrial membrane viscosity, accelerated Ca^{2+} uptake, and increased susceptibility to mitochondrial permeability transition. Reduced the development of left ventricular hypertrophy and dysfunction. Treatment with EPA and DHA prevented tetralinoloyl cardiolipin depletion, LV hypertrophy, and abnormal genes expression with pressure overload; these effects are reversed with a high fat diet. Attenuation of pressure overload induced LV hypertrophy was associated with elevated plasmas levels of adiponectin.[444, 446-449]
	4 – Low Protein	4 – Heart-to-body weight ratio was decreased; TNF-alpha levels were elevated; transcript levels for genes involved in calcium signaling and metabolism were decreased.[450]
High Caloric, Balanced or Unbalanced	1- High Fat diet (60+ % fat) a) No cardiac dysfunction	1a) High fat diet did not worsen cardiac hypertrophy or left ventricular dysfunction despite changes in fat mass, insulin, and leptin. High fat diet changes adiposity and glucose disposal, but does not necessarily lead to cardiac dysfunction.[451, 452]
	b) Cardiac dysfunction	1b) High fat diet feeding induced early stage cardiomyopathy due to impaired calcium homeostasis; seems to be age-dependent as old cardiomyocytes are not able to handle the fatty acid load as well and accumulate lipid intermediates; cardiac mitochondria are unable to

		properly use pyruvate.[453-455]
	2- Western diet (High Carb, High fat diet, 45%)	2 – Inflammation and oxidative stress which led to left ventricular hypertrophy, glucose intolerance, and fibrosis; this is due to maladaptive tissue remodeling[456-458]
	3- High Carb diet (70%)	3 – A high carbohydrate diet may confer cardioprotection despite inducing obesity.[459]
	4- High Protein diet	4 – Does not affect cardiac mass, left ventricular function, ejection fraction, or mitochondrial oxidative capacity; however, does affect survival, and underlines an age-dependent effect.[460, 461]
Pharmacologic Reagents	1 – WY-14,643	1 – PPARalpha leads to increase in fatty acid oxidation which increases expression of MCD (malonyl-CoA dehydrogenase); this attenuates pressure overload induced cardiac hypertrophy. Increased levels of cardiac UCP3.[462, 463]
	2 – Streptozotocin	2 – Streptozotocin decreased glucose metabolism and increased fatty acid metabolism proteins; decreased cardiac glycolysis and PDH and induced either Type 1 or Type 2 diabetes depending on the dose administered.[244]
	3 - Naringin	3 – Improved cardiac function without changing body weight. Used to reverse the left ventricular hypertrophy and fibrosis experienced in rats treated with high carbohydrate, high fat diet.[457]
Hemodynamic Stress	1- Pressure Overload a) High Fructose	1a) - Increased LV mass, decreased ejection fraction; increase in cardiac mass and contractile dysfunction.[441]
	b) High PUFA	1b) - Decreased mitochondrial membrane viscosity, accelerated Ca^{2+} uptake, and increased susceptibility to mitochondrial permeability transition. Reduced the development of left ventricular hypertrophy and dysfunction. Treatment with EPA and DHA prevented tetralinoloyl cardiolipin depletion, LV hypertrophy, and abnormal genes expression with pressure overload; these effects are reversed with a high fat diet. Attenuation of pressure overload induced LV hypertrophy was associated with elevated plasmas levels of adiponectin.[444, 446-449]
	2- Exercise	2 – Swimming exercise improved glucose homeostasis and decreased leptin, resistin, and HOMA-IR index; swimming exercise also ameliorates left ventricular dysfunction.[442]
Ischemia/Reperfusion	1- With PTP (progressive thermopreconditioning)	1 – Increased infarct size, increased ER stress, apoptosis, and autophagy markers; PTP treatment significantly restored cardiac microcirculation, decreased oxidative stress, ER stress, apoptosis, autophagy, and infarct size.[464]
Hormonal Imbalance	1- Leptin	1 – Leptin-treated rats at 5 months have impaired

		contractile function (altered left ventricular systolic function). Hyperleptinemia permanently influences blood pressure and cardiac structure and function.[465]

FIGURE A1

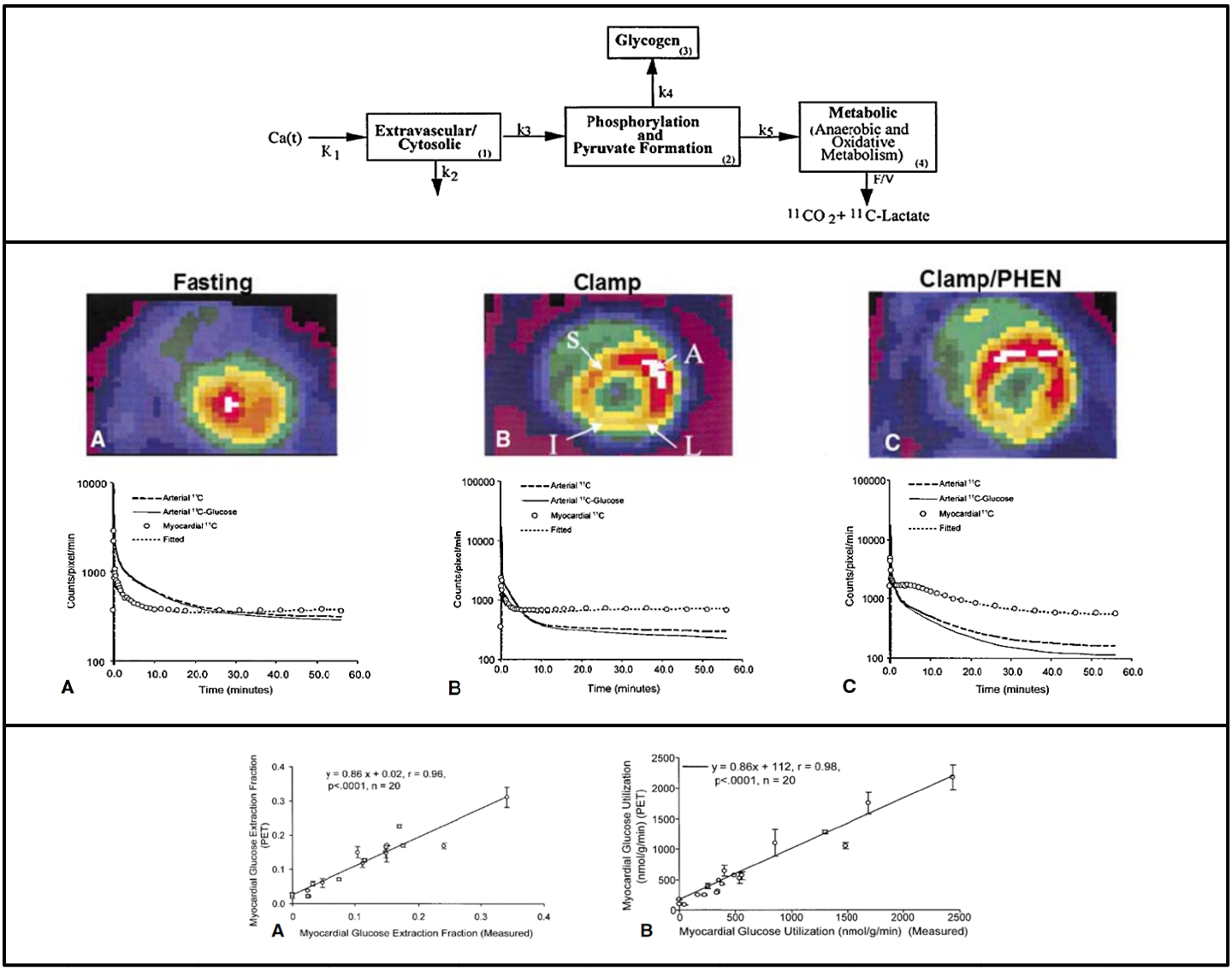

Glycogen (3)
k4
Ca(t)
K1
Extravascular/ Cytosolic (1)
k3
Phosphorylation and Pyruvate Formation (2)
k5
Metabolic (Anaerobic and Oxidative Metabolism) (4)
k2
F/V
11CO2+ 11C-Lactate
Fasting
Clamp
Clamp/PHEN
A
B
C
S
A
I
L
Arterial 11C
Arterial 11C-Glucose
Myocardial 11C
Fitted
Counts/pixel/min
Time (minutes)
y = 0.86 x + 0.02, r = 0.96, p<.0001, n = 20
Myocardial Glucose Extraction Fraction (PET)
Myocardial Glucose Extraction Fraction (Measured)
y = 0.86x + 112, r = 0.98, p<.0001, n = 20
Myocardial Glucose Utilization (nmol/g/min) (PET)
Myocardial Glucose Utilization (nmol/g/min) (Measured)

FIGURE A2

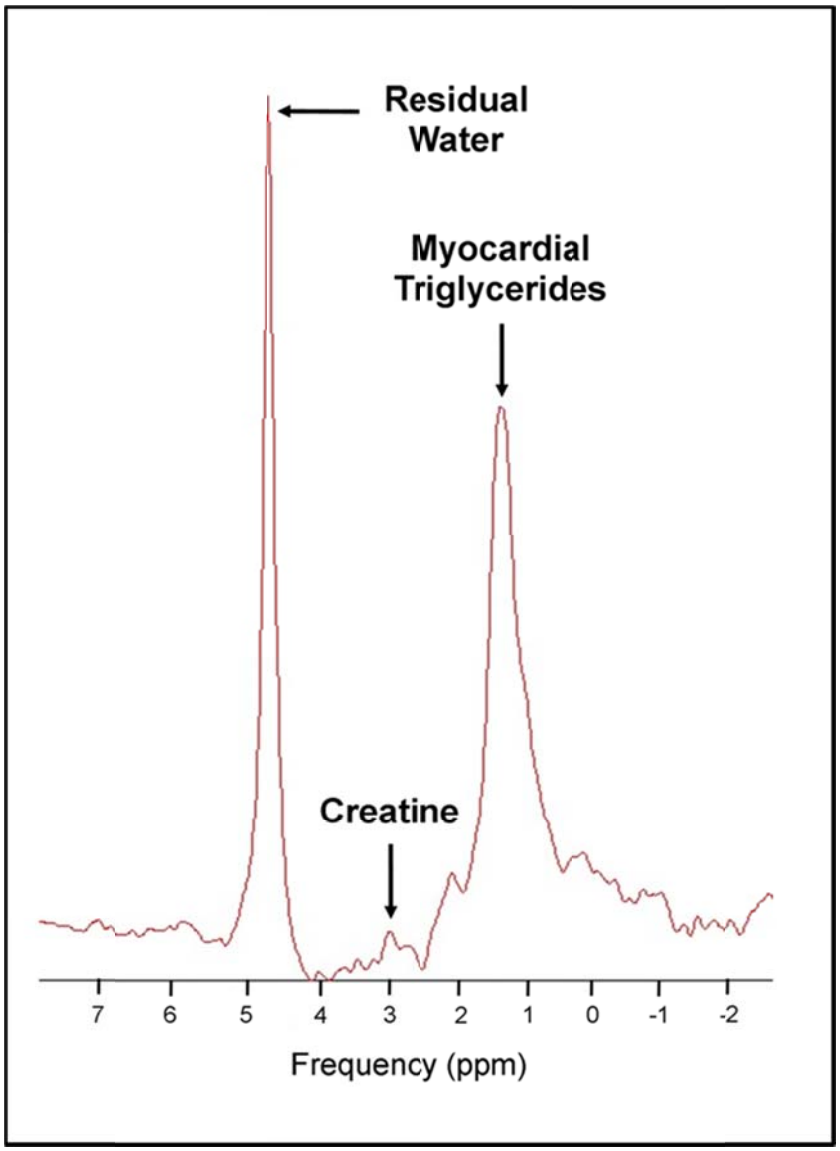
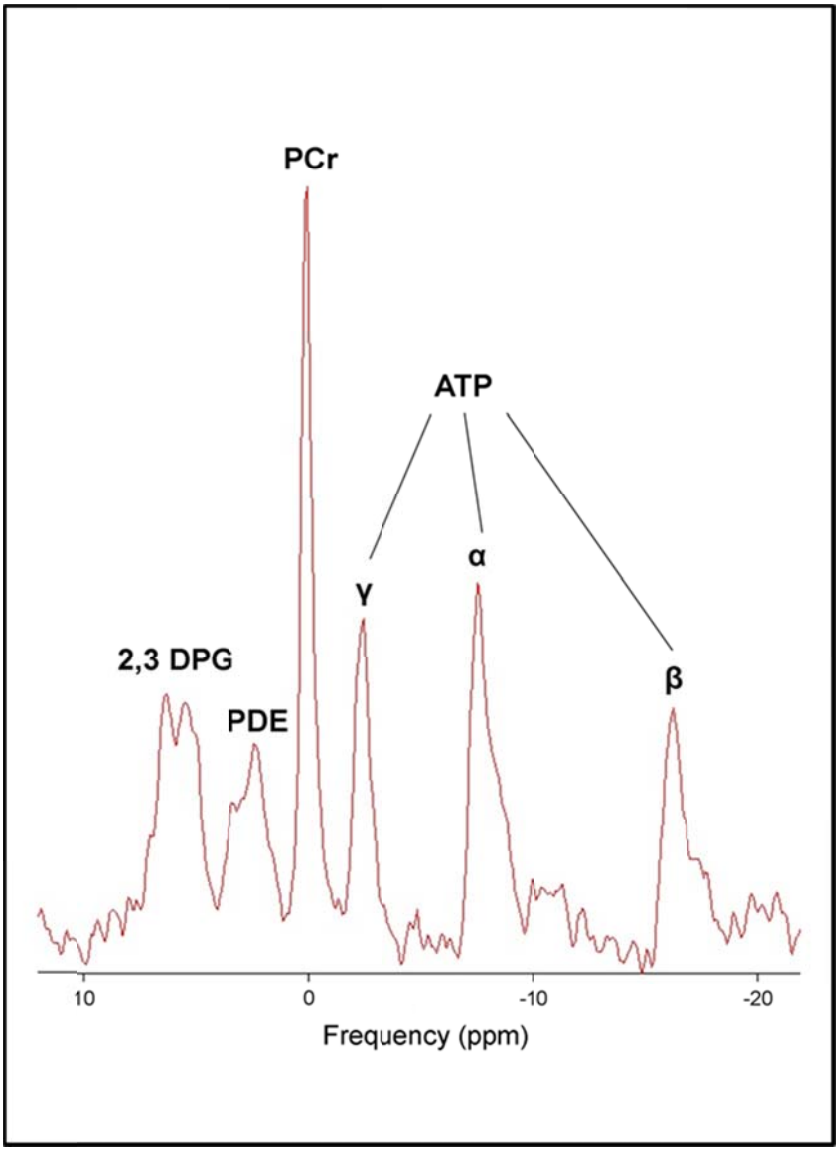

Residual
Water
Myocardial
Triglycerides
Creatine
7
6
5
4
3
2
1
0
-1
-2
Frequency (ppm)

FIGURE A3



Appendix: Legends to Figures

Figure A1: **Measuring Myocardial Glucose Utilization with 1-^{11}C-glucose.**

Top: The kinetics of glucose metabolism are defined by a 4-compartment model that represents a simplification of myocardial glucose metabolism into key metabolic and rate-limiting steps. The various rate constants K_1-k_5 represent the transfer of the radiotracer between compartments. Solution of the differential equations describing the mass transfer of the radiotracer from blood to the different compartments and estimation of the model rate constants and geometric corrections of spillover of activity from blood to myocardium are performed with the use of well-established numerical methods that incorporate the information from the blood (corrected for radiolabeled metabolites) and myocardial time activity curves. Myocardial glucose utilization (in nanomoles per gram per minute) is then calculated from estimated turnover rates and glucose plasma levels. **Middle:** Myocardial 1–^{11}C–glucose images obtained 40 to 60 minutes after radiotracer injection in dogs studied during either fasting, hyperinsulinemic-euglycemic clamp (Clamp) at rest, or hyperinsulinemic-euglycemic clamp with phenylephrine (Clamp/PHEN). Images are displayed in the short-axis view. *S*, Septum; *A*, anterior; *L*, lateral; *I*, inferior. Color scale: *white*, highest counts; *blue*, lowest counts. Corresponding blood and myocardial time activity curves to images A-C. The corresponding arterial blood and myocardial (measured and fitted) curves are shown below. The ^{11}C-activity blood curve represents 1-^{11}C-glucose and its metabolites ^{11}C-lactate and $^{11}CO_2$, whereas the ^{11}C-glucose blood curve represents only 1-^{11}C-glucose and used for the modeling. The varying shapes of the myocardial time activity curves reflect the different levels of myocardial glucose metabolism. **Bottom:** Correlation between PET-derived measurements of myocardial glucose extraction fraction (A) and myocardial glucose utilization rates (B) and directly measured values. *Open circles* represent mean values

for the various interventions, and *error bars* represent SD of the mean. (Reproduced with permission of Mosby, Inc.)[466]

Figure A2: ^{31}P-MR spectrum from the human heart. Please see the section entitled **^{31}P and ^{1}H-MRS (NMR)** for more details.

Figure A3: ^{1}H-MR spectrum from the human heart. Please see the section entitled **^{31}P and ^{1}H-MRS (NMR)** for more details.

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