CALL FOR PAPERS | *Technology Development for Physiological Genomics*Dynamic phosphometabolomic profiling of human tissues and transgenic models by ^{18}O -assisted ^{31}P NMR and mass spectrometryEmirhan Nemutlu,^{1,3} Song Zhang,¹ Anu Gupta,¹ Nenad O. Juranic,² Slobodan I. Macura,² Andre Terzic,¹ Arshad Jahangir,¹ and Petras Dzeja¹¹Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics,²Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, and Scottsdale, Arizona; and ³Department of Analytical Chemistry, Faculty of Pharmacy, University of Hacettepe, Ankara, Turkey

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Nemutlu E, Zhang S, Gupta A, Juranic NO, Macura SI, Terzic A, Jahangir A, Dzeja P. Dynamic phosphometabolomic profiling of human tissues and transgenic models by ^{18}O -assisted ^{31}P NMR and mass spectrometry. *Physiol Genomics* 44: 386–402, 2012. First published January 10, 2012; doi:10.1152/physiolgenomics.00152.2011.—Next-generation screening of disease-related metabolomic phenotypes requires monitoring of both metabolite levels and turnover rates. Stable isotope ^{18}O -assisted ^{31}P nuclear magnetic resonance (NMR) and mass spectrometry uniquely allows simultaneous measurement of phosphometabolite levels and turnover rates in tissue and blood samples. The ^{18}O labeling procedure is based on the incorporation of one ^{18}O into P_i from $[\text{O}^{18}]\text{H}_2\text{O}$ with each act of ATP hydrolysis and the distribution of ^{18}O -labeled phosphoryls among phosphate-carrying molecules. This enables simultaneous recording of ATP synthesis and utilization, phosphotransfer fluxes through adenylate kinase, creatine kinase, and glycolytic pathways, as well as mitochondrial substrate shuttle, urea and Krebs cycle activity, glycogen turnover, and intracellular energetic communication. Application of expanded ^{18}O -labeling procedures has revealed significant differences in the dynamics of G-6-P- $[\text{O}^{18}]$ (glycolysis), G-3-P- $[\text{O}^{18}]$ (substrate shuttle), and G-1-P- $[\text{O}^{18}]$ (glycogenolysis) between human and rat atrial myocardium. In human atria, the turnover of G-3-P- $[\text{O}^{18}]$, which defects are associated with the sudden death syndrome, was significantly higher indicating a greater importance of substrate shuttling to mitochondria. Phosphometabolomic profiling of transgenic hearts deficient in adenylate kinase (AK1 $^{-/-}$), which altered levels and mutations are associated to human diseases, revealed a stress-induced shift in metabolomic profile with increased CrP- $[\text{O}^{18}]$ and decreased G-1-P- $[\text{O}^{18}]$ metabolic dynamics. The metabolomic profile of creatine kinase M-CK/ScCKmit $^{-/-}$ deficient hearts is characterized by a higher G-6- $[\text{O}^{18}]$ P turnover rate, G-6-P levels, glycolytic capacity, γ/β -phosphoryl of GTP- $[\text{O}^{18}]$ turnover, as well as β - $[\text{O}^{18}]\text{ATP}$ and β - $[\text{O}^{18}]\text{ADP}$ turnover, indicating altered glycolytic, guanine nucleotide, and adenylate kinase metabolic flux. Thus, ^{18}O -assisted gas chromatography-mass spectrometry and ^{31}P NMR provide a suitable platform for dynamic phosphometabolomic profiling of the cellular energetic system enabling prediction and diagnosis of metabolic diseases states.

metabolomics; phosphometabolomics; phosphotransfer; metabolite turnover; oxygen-18 stable isotopes; metabolic labeling; energy metabolism; system bioenergetics; mitochondria; adenylate kinase; cre-

atine kinase; gas chromatography; ^{31}P nuclear magnetic resonance; glycolysis; glycogenolysis

METABOLOMIC PROFILING OF DISEASE STATES mandates a comprehensive and simultaneous systematic fingerprinting of multiple metabolites. These are to be identified and quantified, along with their cellular and systemic variations in response to diseases, drugs, toxins, and lifestyle, as well as in the context of genetic or environmental challenges (11, 21, 68, 69, 81, 83, 123, 126). Analytical platforms developed for metabolomics studies allow for the screening of hundreds of metabolites from complex biological samples with analytical precision, comprehensiveness, and sample throughput (34, 79, 84, 120, 126). The physicochemical diversity of metabolites, from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and nonamino organic acids, hydrophobic lipids, and complex natural products necessitates application of different complementary analytical techniques (21, 120, 123). Currently no single platform fulfills all of the requirements as a global metabolite profiling tool. Application of advanced and information-rich spectroscopic techniques is essential for generation of metabolic profiles required for metabolomic studies (80). The main spectroscopic techniques in metabolomic studies are nuclear magnetic resonance (NMR) spectroscopy (^1H , ^{31}P , ^{13}C , ^{17}O , and others) and mass spectrometry [MS; direct infusion or combined with gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE)]. Both techniques can give extensive structural and conformational information on multiple chemical classes in a single procedure; however, they have different analytical strengths and weaknesses (11, 80, 84).

Characterization of metabolic phenotypes requires the knowledge not only of metabolite levels but also of their turnover rates from which metabolic fluxes and, therefore, the dynamic state of a metabolic system can be determined (38, 55, 65, 73, 101). As many metabolites are present in low concentrations and associated with high flux/turnover rates through the metabolite pools, significant changes in metabolic flux could occur without apparent changes in metabolite concentrations (77, 97). Therefore, dynamic metabolomic profiling and flux measurements are essential for comprehensive understanding of metabolic phenotypes (27, 43, 65, 77, 103, 123). Stable isotope tracer-based metabolomic technologies allow simultaneous determination of metabolite levels and their turn-

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over rates with subsequent evaluation of metabolic network dynamics (38, 41, 55, 97, 100, 101). For example, ^{13}C labeling is widely used to track turnover of the carbon backbone of metabolites and label propagation through metabolic networks (49, 94, 131). This technique alone, however, does not allow the acquisition of a full picture of metabolic dynamics and of the status of the cellular energetic system. ^{18}O isotopes are suitable to follow cellular phosphorus turnover and metabolic dynamics of phosphoryls in energetically and signal transduction important biomolecules as well as labeled molecule distribution through phosphotransfer networks (26, 31, 99–102, 114, 134). ^{18}O is a natural and stable isotope of oxygen. When tissue or cells are exposed to media containing water with a known percentage of ^{18}O , $[\text{}^{18}\text{O}]\text{H}_2\text{O}$ rapidly equilibrates with the cellular water, and ^{18}O from water is incorporated into cellular phosphate metabolites proportionally to the rate of the involved enzymatic reactions (31). The percentage of ^{18}O incorporation into phosphate metabolites of interest can be determined by ^{31}P NMR or mass spectrometry (Fig. 1) (44, 101, 135). Incorporation of ^{18}O into phosphoryls induces an isotope shift in the ^{31}P NMR spectrum due to differences in the shielding effects of ^{16}O vs. ^{18}O on the ^{31}P nucleus as well as a shift in the mass spectrum of phosphoryl-containing ions (Fig. 1, B and C) (26, 58, 101). A percentage of incorporated ^{18}O into metabolite phosphoryls calculated from the induced isotope shift in ^{31}P NMR spectra (Fig. 2A) and from GC-MS data can be employed to determine the turnover rates and phosphotransfer flux through specific energetic circuits (Fig. 2B).

The ^{18}O labeling procedure is based on the incorporation of one ^{18}O atom, from $[\text{}^{18}\text{O}]\text{H}_2\text{O}$, into P_i with each act of ATP hydrolysis and the subsequent distribution of ^{18}O -labeled phosphoryls among other phosphate-carrying molecules (Fig. 2B). In conjunction with ^{31}P NMR spectroscopy and MS, the ^{18}O labeling procedure provides a versatile methodology for simultaneous measurement of metabolite levels and metabolic fluxes through phosphotransfer systems allowing characterization of different energetic pathways (44, 46, 65, 99–102, 133, 134) (Fig. 2). This includes simultaneous recordings of ATP synthesis and utilization, phosphotransfer fluxes through adenylate kinase, creatine kinase (CK), and glycolytic pathways, as well as mitochondrial Krebs cycle activity, glycogen turnover and intracellular energetic communication (Fig. 2). An additional advantage of the ^{18}O methodology is that it can measure almost every phosphotransfer reaction taking place in the cell, including cAMP, cGMP, and AMP turnovers and their metabolically active pool sizes (30, 31, 100, 121). The ^{18}O -phosphoryl labeling procedure detects only newly synthesized molecules containing phosphoryls. Their turnover rates and net fluxes through individual metabolic pathways are proportional to the percentage of incorporated ^{18}O (101, 106, 133). Theoretically, up to one-third of all metabolites containing phosphorus (93) and their turnover rates can be quantified using high-resolution ^{31}P NMR spectroscopy and MS. Thus, this technology permits determination of phosphometabolites and multiple phosphotransfer fluxes within metabolic networks.

All metabolomic studies result in complex multivariate data sets that require visualization software and chemometric methods for interpretation. The aim of these procedures is to produce biochemically based fingerprints that are of diagnostic or other stratification value, and to identify potentially complex

sets of biomarkers supporting diagnosis or annotation (5, 11, 25, 52, 92). Here, multivariate data sets obtained from different analytical techniques and ^{18}O -labeling ratios were combined and interpreted using principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and other chemometric techniques to extract latent metabolic information, and to enable sample classification and biomarker discovery.

In this report, we describe the principles and methodology of metabolic profiling and analysis of phosphometabolite turnover rates using stable isotope ^{18}O -assisted ^{31}P NMR and MS. This advanced phosphometabolomic platform is a valuable tool in studies of intact muscle system bioenergetics and phosphotransfer networks, and it is unique for measurements of intracellular energetic communication and metabolic signal dynamics. Basic concepts of ^{18}O labeling are explained and illustrated by data from phosphometabolomic studies of human and rat myocardium and transgenic models of genetic deficiencies. A special focus is placed on sample preparation, measurements, and calculations of phosphometabolite turnover rates and multivariate data analyses.

METHODOLOGY

Phosphometabolomic Platform

Phosphorous is an essential element indispensable to life activity, such as genetic inheritance, signal transduction, metabolism, and energy conversion (110). Phosphate is the most common fragment by the frequency of occurrence in the metabolome of living organisms (93). In the Human Metabolome Database (<http://www.hmdb.ca/>) there are 744 compounds containing “phospho” and 419 with “phosphate” in their structures from of total 8,536 metabolites. Origins of comprehensive analysis of phosphorus-containing metabolites can be traced to Besman’s phosphate analyzer where ^{32}P labeling, chromatographic separation, and quantification of phosphometabolites were performed (13). Most of phosphorus-containing metabolites are highly polar, and their separation and analysis represent a challenge. Phosphometabolites can be measured simultaneously by several analytical techniques, including ^{31}P NMR, LC-MS, GC-MS, CE-MS, and HPLC (3, 110, 117). Although these methods are generally successful in determining the concentration of metabolites, each has its own limitation; thus, it is not possible to measure all phosphometabolites using only one approach. For example, sugar phosphates are best separated by GC-MS (34), while phospholipids with ^1H and ^{31}P -NMR (48) and nucleotides with LC (28).

We have established an expanded dynamic phosphometabolomic platform (Fig. 1) that includes ^{18}O -assisted GC-MS, ^{18}O -assisted ^{31}P NMR, ^1H NMR, and HPLC. Also we are developing 2D ^{31}P NMR and LC-MS-MS methods for quantification of ^{18}O -labeling of mono- or oligo-phosphometabolites (3, 67). The metabolic ^{18}O -assisted GC-MS technology, which originally was developed in Nelson Goldberg’s laboratory at the University of Minnesota (30, 133–135), allows separation and quantitation of $^{18}\text{O}/^{16}\text{O}$ isotope ratios in phosphoryl metabolites with a molecular mass <500 Da. Higher molecular weight phosphates and oligo-phosphates, such as ATP or GTP, can be analyzed after enzymatic transfer of corresponding phosphoryls to glycerol (46, 134). Pioneered by Mildred Cohn and further developed at Mayo Clinic, ^{18}O -assisted ^{31}P NMR technique uses ^{18}O -induced shift in ^{31}P NMR spectra to determine the percentage of ^{18}O -labeling of metabolite phosphoryls (26, 67, 100, 101).

The ^{18}O -labeling technology, which has been used for analyses of enzymatic mechanisms *in vitro* (26, 58), is adapted and developed for tracking phosphoryl metabolic dynamics in intact tissues (100, 101). It was ^{18}O labeling experimentation that led to resolving the rotational catalysis mechanism of F_0F_1 -ATP synthase underlying the Nobel Prize for Paul Boyer (19, 58). The critical

Stable ^{18}O isotope-based phosphometabolomic platform

Fig. 1. Stable isotope ^{18}O -based analytical platform for phosphometabolite analysis and phosphometabolomic fingerprinting of metabolophenotypes. Combination of ^{18}O -assisted gas chromatography-mass spectrometry (GC-MS) and ^{31}P nuclear magnetic resonance (NMR) with ^1H NMR and HPLC provides a powerful platform for dynamic phosphometabolomic profiling of energetic and signaling processes and network analysis cellular bioenergetic systems. **A**: schematic representation of workflow during ^{18}O -labeling procedure and sample preparation. **B**: analysis of ^{18}O -incorporation into metabolite phosphoryls using GC-MS. **C**: measurements of ^{18}O -labeling of phosphometabolites by ^{18}O -induced shift in ^{31}P NMR spectra; a typical ^{31}P NMR spectrum of major phosphometabolites in heart extract and ^{18}O -induced shift in ^{31}P NMR spectra of γ -ATP. **D**: metabolite analysis using HPLC and ^1H NMR. **E**: univariate and multivariate metabolomic data analyses.

advantage of ^{18}O -assisted ^{31}P NMR technique is that it does not require prior metabolite separation and derivatization; it is stable and quantitative and allows simultaneous single-run recordings of multiple metabolite phosphoryls and of separate phosphoryls within one molecule such as α -, β -, and γ -phosphoryls of ATP (Fig. 2A) (100, 101). However, compared with GC-MS, ^{18}O -assisted ^{31}P NMR is less sensitive and requires a larger amount of

the sample and a longer data acquisition time. ^1H NMR in our studies is used as a complementary technology for quantification of phosphometabolite levels in tissue extracts and biological fluids (100). HPLC using ion-exchange, reversed-phase, hydrophobic and hydrophilic interaction chromatography is a versatile technique for separation and quantification of major phosphometabolite classes (Fig. 1) (46, 101, 134). The use of triethylammonium

A Phosphometabolite dynamics by ^{18}O -assisted ^{31}P NMR

Fig. 2. Principles of ^{18}O -labeling methodology for dynamic metabolomic profiling. A: nondestructive phosphometabolite and ^{18}O -labeling analysis using ^{31}P NMR spectroscopy. Metabolic flux-dependent ^{18}O labeling results in induced shift in ^{31}P NMR spectra of phosphoryl containing metabolites. ^{18}O assisted ^{31}P NMR spectra of ^{18}O -labeled P_i , CrP, G-6-P, γ -ATP, β -ATP, and α -ATP in rat heart extract (red line indicates unlabeled, while green line shows ^{18}O -labeled species). Incorporation of ^{18}O induces an isotopic shift in ^{31}P NMR spectra of phosphoryl contained metabolites. ^{16}O , $^{18}\text{O}_1$, $^{18}\text{O}_2$, $^{18}\text{O}_3$, and $^{18}\text{O}_4$ designate phosphoryls containing 0, 1, 2, 3, and 4 atoms of ^{18}O . B: schematic representation of ^{18}O -labeling reactions of different metabolites for comprehensive characterization of cellular energetic system and distribution of multiple phosphotransfer fluxes.

B

bicarbonate (TEAB) buffer, introduced by Smith and Khorana (112) is preferential since the volatility of TEAB facilitates sample recovery after HPLC chromatographic separation and makes it suitable for MS analysis of phosphometabolites.

^{18}O Metabolic Labeling

When tissue or cells are exposed to media containing known percentages (20–30%) of ^{18}O , $^{18}\text{O}[\text{H}_2\text{O}]$ rapidly equilibrates with cellular water and ^{18}O from water is transferred to cellular phosphate

metabolites proportionally to the rate of enzymatic reactions involved (Fig. 2B). The following reactions and cell energetic parameters can be measured:

1. $\gamma\text{ATP} + [^{18}\text{O}] \text{H}_2\text{O} \rightarrow [^{18}\text{O}] \text{P}_i + \text{ADP}$ (ATP hydrolysis)
2. $[^{18}\text{O}] \text{P}_i + \text{ADP} \rightarrow [^{18}\text{O}] \gamma\text{ATP}$ (ATP synthesis)
3. $[^{18}\text{O}] \gamma\text{ATP} + \text{Cr} \rightarrow [^{18}\text{O}] \text{CrP} + \text{ADP}$ (CK-catalyzed phosphotransfer)
4. $[^{18}\text{O}] \gamma\text{ATP} + \text{AMP} \rightarrow [^{18}\text{O}] \beta\text{ADP} + \text{ADP} \rightarrow [^{18}\text{O}] \beta\text{ATP} + \text{AMP}$ (AK-catalyzed phosphotransfer)

5. $[^{18}\text{O}]\gamma\text{ATP} + \text{glucose} \rightarrow \text{glucose-6-}[^{18}\text{O}]\text{P} + \text{ADP}$ (glycolysis, hexokinase-catalyzed phosphotransfer)
6. $\text{glucose-6-}[^{18}\text{O}]\text{P} \leftrightarrow \text{glucose-1-}[^{18}\text{O}]\text{P}$ (PGM, glycogen synthesis/glycogenolysis)
7. $\text{Glycogen (n residues)} + [^{18}\text{O}] \text{P}_i \rightarrow \text{Glycogen (n-1 residues)} + \text{G-1-}[^{18}\text{O}]\text{P}$ (glycogenolysis)
8. $\text{Dihydroxyacetone } [^{18}\text{O}]\text{phosphate} \rightarrow \text{Glycero-3-}[^{18}\text{O}]\text{P}$ (GPDH, substrate shuttle)
9. $[^{18}\text{O}] \text{P}_i/[^{18}\text{O}]\gamma\text{ATP}$ ratio (energetic communication, ATP utilization $\leftrightarrow$ synthesis sites)
10. $[^{18}\text{O}] \gamma\text{ATP} + \text{Adenosine} \rightarrow [^{18}\text{O}]\alpha\text{AMP} + \text{ADP}$ (adenosine kinase, nucleotide synthesis)
11. $[^{18}\text{O}] \gamma\text{ATP} \rightarrow \text{AMP} + [^{18}\text{O}] \text{PP}_i$ (synthetic reactions, DNA, RNA)
12. $[^{18}\text{O}]\gamma\text{ATP} + \text{GMP} \rightarrow [^{18}\text{O}]\beta\text{GDP} + \text{ADP}$ (GK-catalyzed phosphotransfer)
13. $[^{18}\text{O}]\gamma\text{ATP} + \text{GDP} \rightarrow [^{18}\text{O}]\gamma\text{GTP} + \text{ADP}$ (NDPK/succinyl-CoA synthase phosphotransfer)
14. $\text{cAMP} + [^{18}\text{O}] \text{H}_2\text{O} \rightarrow [^{18}\text{O}]\alpha\text{AMP}$ (cAMP phosphodiesterase, signal transduction)
15. $\text{L-arginine} + [^{18}\text{O}] \text{H}_2\text{O} \rightarrow \text{urea}[^{18}\text{O}] + \text{L-ornithine}$ (ARG, urea cycle, redox, NO balance)

The rates of sequential enzymatic reactions between P_i , $\gamma\text{-ATP}$, and creatine phosphate (CrP) are high and, upon ^{18}O labeling, display exponential kinetics with saturation occurring within 2 min (Fig. 3A) (99, 100). Therefore, labeling of metabolites should be performed within the initial linear phase (0–1 min) of the ^{18}O -labeling curve, while for $\beta\text{-ADP}$ and $\beta\text{-ATP}$, which have lower turnover rates, labeling can be performed within a 5 min time window. At the end of the desired exposure, cell metabolism is instantaneously stopped by immersing cells or tissue into liquid N_2 or by freeze-clamping with liquid N_2 -cooled tongs.

Heart perfusion and ^{18}O phosphoryl labeling. Hearts from hep- arinized (50 U ip) and anesthetized (75 mg/kg pentobarbital sodium ip) wild-type or transgenic mice were excised and retrogradely perfused with a 95% O_2 -5% CO_2 -saturated Krebs-Henseleit (K-H) solution (in mM: 118 NaCl, 5.3 KCl, 2.0 CaCl_2 , 19 NaHCO_3 , 1.2 MgSO_4 , 11.0 glucose, 0.5 EDTA; 37°C) at a perfusion pressure of 70 mmHg. Hearts were paced at 400 beats/min. Hearts were perfused for 30 min and then subjected to labeling with ^{18}O , which is introduced for 30–60 s with the K-H buffer supplemented with 20–30% of ^{18}O -labeled H_2O (Isotec, Sigma), and then hearts are immediately freeze-clamped. The frozen hearts were pulverized under liquid N_2 , and extracted in a solution containing 0.6 M HClO_4 and 1 mM EDTA. Extracts were neutralized with 2 M KHCO_3 and used to determine the extent of ^{18}O incorporation into metabolite phosphoryls (44, 102).

Atrial myocardium ^{18}O phosphoryl labeling. Right atrial appendage tissues from seven patients, free of atrial pathology and undergoing coronary artery bypass surgery, were subjected to metabolite ^{18}O -labeling. Experiments were conducted with the understanding and the consent of patients, and were approved by the Mayo Clinic Institutional Review Board. Right atrial appendages isolated from patients were exposed to K-H solution enriched with 30% of $[^{18}\text{O}]\text{H}_2\text{O}$ for 1 min and then freeze-clamped into liquid nitrogen and stored for ^{18}O labeling and biochemical analysis. For comparison appendages from rats were used. The investigation conformed to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. Atrial tissues were removed from surgically excised hearts from anesthetized (sodium pentobarbital 50 mg/kg ip injection) male rats, which were maintained on a standard chow diet and housed in a controlled environment for 1 wk before death. Immediately after harvest, atrial tissue was exposed to K-H solution enriched with 30% $[^{18}\text{O}]\text{H}_2\text{O}$ for 1 min and then freeze-clamped into liquid nitrogen for ^{18}O -labeling and biochemical analysis. Freeze-clamped atrial samples were

pulverized in mortar with liquid nitrogen and extracted in a solution containing 0.6 M HClO_4 and 1 mM EDTA. Extracts were neutralized with 2 M KHCO_3 and used for analysis of the ^{18}O -labeling by HPLC and MS. The ^{18}O -enrichment of phosphoryls in glycerol-3-phosphate (G-3-P), glucose-6-phosphate (G-6-P), and P_i was determined with a GC-MS in the select ion-monitoring mode. The percentage of phosphoryl oxygens replaced by ^{18}O was calculated according to the formula $[\%^{18}\text{O}_1 + 2(\%^{18}\text{O}_2) + 3(\%^{18}\text{O}_3)]/3(\%^{18}\text{O}[\text{H}_2\text{O}])$ for G-3-P and G-6-P and $[\%^{18}\text{O}_1 + 2(\%^{18}\text{O}_2) + 3(\%^{18}\text{O}_3) + 4(\%^{18}\text{O}_4)]/4(\%^{18}\text{O}[\text{H}_2\text{O}])$ for P_i as described previously (44, 133).

GC-MS Analysis of ^{18}O -labeling

^{18}O labeling ratios of metabolite monophosphates [such as G-3-P, G-6-P, and glucose-1-phosphate (G-1-P)] were evaluated using GC-MS after purification with HPLC, because of their low concentration per sample (Fig. 1B). Although P_i concentration in the sample is high, it must be separated from other phosphate-containing metabolites as some of them are unstable during GC-MS analysis and metabolites such as CrP or GA-3-P are readily degraded and could liberate P_i , which would interfere with free P_i labeling measurements. Therefore, samples were fractionated and concentrated using HPLC.

Cellular phosphometabolites were purified and quantified with HPLC (Fig. 1D) using a Mono Q HR 5/5 ion-exchange column (Pharmacia Biotech) and 1 M TEAB buffer (pH 8.8, adjusted with CO_2) gradient elution (0.5–90% within 25 min) at 1 ml/min flow rate (44, 46, 95). From each sample seven fractions were collected. The first fraction contained G-6-P, G-3-P, G-1-P, and CrP and fractions from second to the seventh contained AMP, P_i , ADP, GDP, ATP, and GTP, respectively. Fractions were dried out using vacuum centrifugation (SpeedVac, Savant) and reconstituted with water. Monophosphates were transferred to GC-MS vials for silylation, while oligophosphates were subjected to enzymatic reactions in Eppendorf tubes to transfer each phosphoryl to glycerol. The γ -phosphoryls of ATP or GTP are transferred to glycerol by glycerokinase, and β -phosphoryls of ATP and ADP are transferred to glycerol by a combined catalytic action of adenylate kinase and glycerokinase. The β -phosphoryls of GTP and GDP are transferred to glycerol by a combined catalytic action of guanylate kinase and glycerokinase. The phosphoryl of CrP is transferred to $\gamma\text{-ATP}$ by CK and then to glycerol with glycerokinase. Samples that contained phosphoryls of $\gamma\text{-ATP}$, $\gamma\text{-GTP}$, $\beta\text{-ATP}$, $\beta\text{-ADP}$, $\beta\text{-GTP}$, GDP, and CrP are transferred to G-3-P and samples with P_i , G-6-P, G-1-P, AMP, and G-3-P are converted to respective trimethylsilyl derivatives using Tri-Sil/BSA (Pierce) as the derivatization agent (44, 100). The ^{18}O enrichments of phosphoryls were determined with GC-MS operated in the selected ion-monitoring mode. GC-MS analysis of G-3-P and G-6-P ^{18}O -labeling is presented in Fig. 1B. The left panel represents GC-MS chromatograms of metabolites, while in the right panel oxygen isotope abundance is shown. Another phosphometabolite G-1-P can be analyzed in the same HPLC fraction too. With this approach in a single run the metabolic dynamics of glycolysis and glycogenolysis and mitochondrial substrate shuttle activity can be monitored. The ^{18}O enrichments of phosphoryls were determined with GC-MS operated in the selected ion-monitoring mode. Mass ions (m/z) of selected metabolites monitored as trimethylsilyl derivatives are given in Table 1. Monophosphates are able to get replaced up to three oxygens with ^{18}O , while P_i and inorganic pyrophosphate (PP_i) can get up to four and seven ^{18}O oxygens, respectively. Mass ions (m/z) of monophosphates corresponding to phosphoryl species of ^{16}O , $^{18}\text{O}_1$, $^{18}\text{O}_2$, and $^{18}\text{O}_3$ were monitored at parent ion (^{16}O) + 2, +4, and +6, respectively (44, 133).

^{31}P NMR Analysis of ^{18}O Incorporation

Samples were precleaned with Chelex 100 resin (Sigma) by incubating for 1 h, supplemented with internal standard for ^{31}P NMR

Tracking dynamics of metabolic pathways

Fig. 3. Analysis of phosphometabolite dynamics in human and rat atrial myocardium. A: kinetics of ^{18}O -labeling of P_i and γ -ATP in rat myocardium indicating dynamics of ATP utilization and ATP synthesis cycles (incorporation of $^{18}\text{O}_1$, $^{18}\text{O}_2$ and $^{18}\text{O}_3$). B: turnovers of G-6-P [^{18}O] (glycolysis), G-3-P [^{18}O] (substrate shuttle), and G-1-P [^{18}O] (glycogenolysis) in rat (top) and human (bottom) atria. C: schematic representation of ^{18}O -labeling reaction sequences in tracking dynamics of glycolytic and glycogenolytic networks and substrate shuttles in the heart.

Table 1. Mass ions (m/z) of selected phosphometabolites that correspond to ^{18}O -labeled phosphoryl species monitored as trimethylsilyl derivatives

	^{16}O	$^{18}\text{O}_1$	$^{18}\text{O}_2$	$^{18}\text{O}_3$	$^{18}\text{O}_4$
P_i	299	301	303	305	307
G-3-P	357	359	361	363	
G-6-P	387	389	391	393	
G-1-P	217	219	221	223	
CrP (G-3-P)*	357	359	361	363	
PEP	369	371	373	375	
$\text{PP}_i^\dagger$	451	453	455	457	459
3-PG	357	359	361	363	
IMP	315	317	319	321	
AMP	315	317	319	321	
R-5-P	315	317	319	321	
γATP (G-3-P)*	357	359	361	363	
βATP (G-3-P)*	357	359	361	363	
αATP (G-3-P)*	357	359	361	363	
βADP (G-3-P)*	357	359	361	363	
αADP (G-3-P)*	357	359	361	363	

*Phosphate labeling was determined as G-3-P after enzymatic transferring it to glycerol. $\dagger\text{PP}_i$ can be labeled up to 7 oxygen atoms. For simplicity, 4 of them are given in the table.

spectroscopy methylene diphosphonate, and concentrated by vacuum centrifugation (SpeedVac, Savant) to a volume of 0.3 ml. Concentrated extracts were filtered (centrifuge filter; 0.22 μm , Millipore) and supplemented with 0.1 ml of D_2O (Isotec) and 0.1 ml 1 mM EDTA. Samples were additionally treated with the Chelex 100 resin by rotation at 4°C for 12 h to eliminate last traces of two-valent cations. Their elimination improves spectral resolution and increases the sample stability during data acquisition (99–102).

^{31}P NMR data acquisition was performed at 202.5 MHz (Avance, Bruker) at ambient temperature as described earlier (102). A typical ^{31}P NMR spectrum of heart extract is shown in Fig. 1C. Incorporation of each ^{18}O induces isotope shifts between 0.0210 and 0.0250 ppm in the ^{31}P NMR spectrum of P_i , CrP, $\gamma\text{-ATP}$, $\beta\text{-ATP}$, $\alpha\text{-ATP}$, $\beta\text{-ADP}$, $\alpha\text{-ADP}$, AMP, PC, G-6-P, G-1-P, and G-3-P. It should be noted that the isotope shift in the spectrum of $\beta\text{-ATP}$ was different for the bridging and nonbridging ^{18}O oxygens, 0.0170 and 0.0287 ppm, respectively (Fig. 2A). Although the ^{18}O -induced isotope shift is rather small it can be visualized and quantified using high-resolution NMR spectroscopy (Fig. 1C). G-6-P exists in an equatorial and an axial form, and therefore the ^{16}O and ^{18}O species of G-6-P are represented with two peaks corresponding to each of the two forms (Fig. 2A). During the integration procedure, bridging and nonbridging forms of $\beta\text{-ATP}$ as well as equatorial and axial forms of G-6-P for particular ^{16}O or ^{18}O species were integrated as a single peak.

Phosphometabolite Analysis by ^1H -NMR

^1H NMR provides a robust and precise method for metabolite quantification including the number of phosphometabolites. ^1H NMR data acquisition was performed at 500 MHz (Bruker, Avance) at ambient temperature and sample spinning at 20 Hz; 128 scans were accumulated under fully relaxed conditions (12.8 s relaxation delay) with a 9 μs (90°) pulse. Chemical shifts were assigned relative to the trimethylsilyl propionate (TSP) signal at 0 ppm. Metabolite levels such as AMP, ATP, ADP, IMP, CrP, glycolytic intermediates, and phospholipids were calculated according to TSP used as internal standard. Metabolites were identified using the Chenomx NMR Suite software, which provides a pattern recognition technique and efficient method for identifying metabolites in biofluids, and confirmed by standard additions.

Data Analysis and Calculations of Phosphoryl Turnover and Phosphotransfer Fluxes

Introduction of ^{18}O water in tissues of interest leads to ^{18}O incorporation into cellular phosphates according to the rate of involved phosphotransfer reactions (see Fig. 2B) (31, 46, 101, 134). This property allows for the tracking of high-energy phosphoryl transfer routes and quantification of respective enzymatic fluxes at different levels of cellular activity (31, 44, 46, 99–102, 134). Up to three ^{18}O atoms can be incorporated in monophosphate (G-3-P, G-6-P, G-1-P, and CrP) and phosphate at a different position in oligophosphate (γ -, β -, and α - for triphosphates and β - and α - for diphosphates) and up to four and seven for P_i and PP_i , respectively. The percentages of ^{16}O , $^{18}\text{O}_1$, $^{18}\text{O}_2$, $^{18}\text{O}_3$, and $^{18}\text{O}_n$ are proportional to integrals of their respective peaks in the ^{31}P NMR spectra or in the GC-MS chromatograms (99–102) (see Figs. 1 and 2). The cumulative percentage of phosphoryl oxygens replaced by ^{18}O in the metabolites was calculated as $[\%^{18}\text{O}_1 + 2(\%^{18}\text{O}_2) + 3(\%^{18}\text{O}_3) + \dots + n(\%^{18}\text{O}_n)]/[n(\%^{18}\text{O in H}_2\text{O})]$ (100, 101), where n is the total number of phosphoryl oxygen sites in the metabolite.

Basic principles and procedures of metabolite turnover rate calculations and metabolic flux estimations from isotope metabolic labeling data were described previously (31, 71, 73, 104, 127, 135, 136). Calculation of $\alpha\text{-AMP}$ or other metabolite turnover time was done using the formula: $p_t(\alpha\text{-AMP}) = (1 - 2^{-N}) \times p([^{18}\text{O}]\text{H}_2\text{O})$, where $p_t(\alpha\text{-AMP})$ is a fraction of ^{18}O -labeled $\alpha\text{-AMP}$ at given time t , N is equal to the number of turnover cycles observed during incubation period, and $p([^{18}\text{O}]\text{H}_2\text{O})$ is fraction of ^{18}O in media water (71, 104, 136). Thus, AK-independent turnover time of the AMP pool can be calculated from the expression: $T = t/N$, where T is the turnover time in s. AK-dependent AMP turnover is calculated using the formula: $dN/dt = \rho[p(\gamma) - p(\beta)]$ where $p(\beta)$ is the fraction of ^{18}O -labeling of adenine nucleotide β -phosphoryls, $p(\gamma)$ is the fraction of ^{18}O -labeling of precursor or adenine nucleotide γ -phosphoryls, and ρ is the rate of ^{18}O -labeling in the nucleotide pool (71, 104). Total AMP turnover (adenylate kinase and nonadenylate kinase mediated) is estimated from the kinetics of AMP α -phosphoryl (nonadenylate kinase) and $\beta\text{-ATP}/\beta\text{-ADP}$ phosphoryl (mediated by adenylate kinase) ^{18}O -labeling (135).

Total cellular ATP turnover can be estimated from the total number of ^{18}O atoms that appeared in the phosphoryl-containing metabolites and orthophosphate (44, 46, 100). The kinetics of ^{18}O -labeled phosphoryl appearance in $\gamma\text{-ATP}$ reflects cellular ATP synthesis rate, while kinetics of P_i ^{18}O -labeling indicates cellular ATPase activity (44). The $\text{P}_i/\gamma\text{-ATP}$ ^{18}O -labeling ratio, an index of intracellular energetic communication (40), is calculated using the amount or percentage of ^{18}O -incorporated into P_i and $\gamma\text{-ATP}$. Incorporation of ^{18}O into P_i and $\gamma\text{-ATP}$ induces very robust multiple shifts in ^{31}P NMR spectra depending on number of oxygens replaced (Fig. 2A). Labeling reaches saturation within 2–5 min, from which the metabolically active pool size of specific metabolite can be determined. At saturation, almost 100% of $\gamma\text{-ATP}$ and CrP and $\sim 80\%$ of P_i are metabolically active (^{18}O labeled). The metabolically active pool size of P_i , $\beta\text{-ADP}$, and $\beta\text{-ATP}$ depends on muscle contraction and ATP turnover rate (133–135). Incorporation of one, two, three, and four atoms of ^{18}O into phosphoryls reflects $\text{P}_i \leftrightarrow \text{ATP}$ cycling between ATP consumption and ATP production sites.

Adenylate kinase phosphotransfer flux is determined from the rate of appearance of ^{18}O -containing β -phosphoryls in ADP and ATP (31, 133, 135). Turnover rates of $\beta\text{-ADP}$ and $\beta\text{-ATP}$, which reflect adenylate kinase activity, are calculated by the procedure described above from the kinetics of $\gamma\text{-ATP}$ (precursor) and $\beta\text{-ATP}$ or $\beta\text{-ADP}$ ^{18}O -labeling (104). The activity of adenylate kinase in intact tissue is a function of both the rate of adenylate kinase catalysis and the kinetics of ^{18}O appearance in $\gamma\text{-ATP}$. The γ -phosphoryl of ATP is transferred to AMP in the adenylate kinase-catalyzed reaction without loss or gain of ^{18}O atoms (133). The flux can be estimated by fitting

metabolic labeling data to kinetic models using Stella (31, 100, 133, 135), CWave (86), FiatFlux (132), FluxSimulator (15), or similar software. To obtain adenylate kinase kinetics and total number of ^{18}O -labeled phosphoryls in β -ADP and β -ATP produced by the adenylate kinase catalysis, precursor γ -ATP labeling kinetics as well as metabolically active metabolite pool size are considered (133, 135). The pool of metabolically active ADP, obtained from labeling studies, is usually larger than free ADP calculated from the CK equilibrium (108, 135) and is in dynamic equilibrium between free and bound states (9, 45). Dynamics of free and bound ADP, which is different from those derived using the CK equilibrium, has been recently demonstrated by ^{31}P NMR saturation transfer (91), suggesting the presence of solid-state biochemistry in enzymatic clusters and cellular energetics. The metabolically active ADP or other phosphometabolite pool size is determined after prolonged (10–20 min) ^{18}O -labeling to establish isotopic equilibrium (135). Best fits to experimental data of kinetic model are obtained using a metabolically active (^{18}O -labeled) pool size of 80–90% for β -ATP and 20–30% for β -ADP (135). Such kinetic analysis, which includes rate of label incorporation, metabolic pool size, precursor labeling, and loss of label from a specific metabolite pool, permits the establishment of the relationship between measured metabolite labeling rate, turnover rate, and estimated metabolic flux through a specific pathway. Knowing such a kinetic relationship, one can estimate the metabolic flux from the labeling data at a specific time point in the initial pseudo-linear labeling curve (46, 133–135).

A similar procedure is used for establishing the relationship between CrP labeling, turnover rate, and CK phosphotransfer flux (46, 134). Since the CrP labeling rate is high and close to γ -ATP, the precursor labeling rate, a gradual inhibition of CK activity is necessary for establishing the relationship between the measured CrP ^{18}O -labeling and the CK metabolic flux (46). The metabolic flux of CrP is equal to the turnover rate times its cellular concentration (133). After such a relationship is determined, the CK metabolic flux can be estimated from the labeling data at a specific time point in the initial pseudo-linear labeling curve (46).

The CK catalytic rates determined by the ^{18}O phosphoryl oxygen exchange analysis differ by more than an order of magnitude with those determined by the ^{31}P NMR saturation transfer technique (16, 44, 74). This difference undoubtedly stems from the different kinetic parameters quantified by these procedures (8, 51). ^{31}P or ^{13}C NMR saturation transfer analysis assesses the total unidirectional exchange rates catalyzed by CK or other enzymes, which reflects enzyme activity in vivo (8, 130), whereas the phosphoryl ^{18}O -labeling measurements assess net phosphoryl flux, i.e., appearance of newly generated molecules of ^{18}O -labeled CrP, which are transferred from mitochondria to ATP utilization sites (41, 46, 133). That ^{31}P NMR saturation transfer measures CK catalytic capacity is supported by the observation that CK overexpression doubles unidirectional exchange rate at the same level of muscle activity and energy consumption (20). Moreover, the sum of unidirectional exchanges in forward and backward CK reactions, which at steady state are catalyzed by separate enzyme molecules, is approximately equal to the total CK activity calculated from kinetic constants and substrate concentrations in vivo (16). The difference in the forward and reverse rates of the CK reaction, signifying net flux, is directly proportional to the rate of ATP turnover (75). It is well established from chemical analysis and isotope labeling that CrP utilization during muscle contraction is directly proportional to the frequency of contraction and ATP/energy usage (7, 12, 17). The difference of the magnitude found between these unidirectional exchange rates and net flux measurements is predictable if phosphoryl transfer by CK catalysis was accomplished, as previously suggested (45), by the mechanism of vectorial ligand conduction along the enzymatic chain or cluster (41). According to this mechanism, this difference would represent the average number of CK-catalyzed equilibration reactions involved in the propagation of a newly synthesized molecule of CrP from its site of generation to an

ATP utilization site. Estimates show that each CrP molecule produced undergoes 50–100 unidirectional forward and backward CK-catalyzed phosphotransfers in transit from mitochondria to ATP consumption sites or ATP membrane sensors in the intact muscles (42, 46).

Using similar procedures, we determined metabolite turnover rates, glycolytic flux, and cytosolic-mitochondrial glycerol phosphate shuttle activity from the rate of appearance of ^{18}O -labeled G-6-P and G-3-P, respectively (65, 100), whereas glycogen flux can be determined from the rate of appearance of ^{18}O -labeled G-1-P. Activity of NDPK/Succinyl-CoA synthase is determined from γ -GTP ^{18}O -labeling, while the β -GTP/GDP ^{18}O -labeling rate indicates guanylate kinase velocity. Thus, the entire network of phosphotransfer reactions can be followed using this technology (Fig. 2B).

Multivariate Statistical Analysis

Multivariate data sets obtained from different analytical techniques and labeling ratios were combined and interpreted with PCA and PLS-DA methods. Initially, the data were examined with PCA scatter plot of the first two score vectors (t1-t2) to reveal the homogeneity of the data, any groupings, outliers, and trends. Then PLS-DA is applied to get additional information, increase the class separation, simplify interpretation, and find potential biomarkers (116, 122). The additional information (significant metabolites in group classification) may be obtained using VIP (variable importance in the projection), loading, and regression coefficients plots. The VIP values (10, 116, 128), a weighted sum of squares of the PLS weight that indicates the importance of the variable to the whole model, were calculated to identify the most important molecular variables for the clustering of specific groups, while the regression coefficient plots of metabolic variables in the PLS-DA model show variable effect on the groups larger coefficient values (positive or negative) and have a stronger correlation with group metabolic profile classification. Examination of the corresponding loading plot indicated those metabolites responsible for the clustering of groups. Metabolites located around the center of the plot do not contribute to the clustering of the patient groups, whereas those in the same geographical region of a sample group in the corresponding score plot are responsible for the separation. Attention must be given to the PLS-DA, because it is a supervised method. Even if the two groups are not different from each other, the method could force separation (125). Therefore the PLS-DA model must be validated. For validation, R^2 (the fraction of variance explained by a component) and Q^2 (the fraction of the total variation predicted by a component) values are considered as measures of goodness of model and model robustness, respectively. The value of Q^2 ranges from 0 to 1 and typically a Q^2 value >0.4 is considered a good model, and those with Q^2 values >0.5 are robust (6, 116). Additionally, the validation of the PLS-DA model can be performed by comparison to the classification statistics of models generated after random permutations of the class matrix. If the model R^2 and Q^2 values are higher than those obtained in random permuted models across all iterations, the method is valid. Calculation of the PCA and PLS-DA model parameters was carried out using SIMCA-P+ (v12.0; Umetrics, Umeå, Sweden) and MetaboAnalyst web browser (128).

RESULTS AND DISCUSSION

Phosphometabolite Turnover Analysis in Human and Rat Atrial Myocardium

Alterations in atrial phospho-bioenergetics may increase a predisposition to electrical instability and mechanical dysfunction but has not been characterized in detail in human and rat myocardium. Our previous studies indicate that phosphotransfer enzyme activities play an important role in maintaining myocardial ATP levels and mechanical function and reducing the duration of atrial fibrillation (23, 24, 39, 76). Other studies

have revealed alterations in the atrial proteome and metabolome associated with fibrillation and arrhythmogenic remodeling, suggesting persistence of metabolic stress and less effective energy utilization (32, 87). Here, simultaneous determination of phosphometabolite levels and their turnover rates were performed in human and rat atrial myocardium to compare dynamic metabolic arrangements of their respective energetic systems. Tracking kinetics of ^{18}O -labeling of P_i and $\gamma\text{-ATP}$ (Fig. 3A) permits determination of the dynamics of ATP utilization and ATP synthesis processes in intact myocardium and the rate of cycling of high-energy phosphoryls between ATPase and ATP synthase sites (incorporation of $^{18}\text{O}_1$, $^{18}\text{O}_2$, and $^{18}\text{O}_3$). Labeling of P_i and $\gamma\text{-ATP}$ is relatively fast, approaching saturation within 2–5 min at $\sim 70\%$ of oxygens replaced by ^{18}O in P_i and $\sim 90\%$ in $\gamma\text{-ATP}$ (Fig. 3A). The ratio between labeling rates of P_i (ATPase compartment) and $\gamma\text{-ATP}$ (ATP synthesis compartment, mostly mitochondrial matrix) - $\text{P}_i[^{18}\text{O}]/\gamma\text{-ATP}[^{18}\text{O}]$ reflects intracellular energetic communication, which is depressed in ischemia-injured myocardium and in hearts of phosphotransfer enzyme and K-ATP channel-deficient transgenic animals (37, 38, 57, 66, 101). Glycolytic and glycogenolytic phosphotransfers can be monitored by measuring rates of appearance of ^{18}O -labeled phosphoryl species in G-6-P and G-1-P (Fig. 3, B and C). In this regard, metabolic networks of glycolytic and glycogenolytic enzymes, in addition to their energy-producing role, have the capability of catalyzing high energy phosphoryl transfer between cellular sites of ATP consumption and ATP generation (35). Glycolytic phosphotransfer plays a critical role in sarcolemmal energetics and metabolic sensing by providing ATP for membrane ATPases and regulating nucleotide ratios in the vicinity or “sensing zone” of metabolic sensors such as K-ATP channels, thus providing a link between heart metabolic and electrical activities (42, 61, 107). The glycerophosphate substrate shuttle plays a crucial role in cellular redox balance and bioenergetics by linking cytosolic metabolic networks to mitochondrial oxidations (105). Mutations in G-3-P dehydrogenase 1-like (GPD1-L) protein, which is highly expressed in the heart, are linked to Brugada syndrome and sudden infant death syndrome (SIDS) (118). In this study, the glycerolphosphate shuttle activity was examined by measuring the rate of appearance of ^{18}O -labeled phosphoryl species in G-3-P (Fig. 3, B and C).

The results demonstrate that selected metabolites and turnover rates of G-6-P (glycolysis), G-3-P (mitochondrial substrate shuttle), and G-1-P (glycogenolysis) are differentially expressed in human and rat atria (Fig. 3, B and C). Human atrial myocardium has higher G-6-P[^{18}O], G-3-P[^{18}O], and G-1-P[^{18}O] turnover rates, indicating vigorous activity of glycolytic, substrate shuttling, redox balance, and glycogenolytic pathways. Higher turnover of G-3-P[^{18}O] indicates a greater importance of redox balance and substrate shuttling to mitochondria. Preliminary data indicate that G-3-P metabolic dynamics is altered in the blood of transgenic animal models (AK1 $^{-/-}$, not shown), indicating that defects in the substrate shuttle and supply of reducing equivalents to mitochondria can be tracked by measuring whole blood metabolite turnover. This is of importance since G-3-P turnover abnormalities and metabolic arrest are linked to human diseases such as sudden death syndrome (118). Beyond translational research and medical diagnostics, G-3-P ^{18}O -labeling technology provides a useful platform in disease biomarker research in Brugada syndrome

and SIDS. G-3-P is also the central metabolite required for phosphoglyceride and triglyceride synthesis, and its dynamics is a valuable indicator of metabolic disturbances (59). Thus, measurements of P_i , $\gamma\text{-ATP}$, G-6-P, G-3-P, G-1-P, and other phosphometabolite levels and turnover rates provide valuable information regarding dynamics of atrial metabolic and energetic signaling processes important in elucidation of disease mechanisms and selection of treatment choices.

Phosphometabolomic Profiling of Transgenic Animal Models: Stress-induced Metabolic Response of AK1 Knockout Hearts

Maintenance of optimal cardiac function requires precise control of cellular nucleotide ratios and high-energy phosphoryl fluxes between mitochondria and ATP utilization sites in myofibrils, sarcoplasmic reticulum and sarcolemma (35, 41, 62, 106). Within the cellular energetic infrastructure, adenylate kinase has been recognized as an important phosphotransfer enzyme that catalyzes adenine nucleotide exchange ($\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}$) and facilitates transfer of both β - and γ -phosphoryls in ATP (35, 36). In this way, adenylate kinase doubles the energetic potential of ATP as a high-energy-phosphoryl carrying molecule and provides an additional energy source under conditions of increased demand and/or compromised metabolic state (44). By regulating adenine nucleotide processing, adenylate kinase has been implicated in metabolic signal transduction and metabolic monitoring (36). Indeed, phosphoryl flux through adenylate kinase has been shown to correlate with functional recovery in the metabolically compromised heart and to facilitate intracellular energetic communication (37, 44, 99, 101). Deletion of the major adenylate kinase AK1 isoform, which catalyzes adenine nucleotide exchange, disrupts cellular energetic economy, and compromises metabolic signal transduction and ischemia-reperfusion response (22, 37, 65, 99, 102). Mutations in adenylate kinase *AK1*, *AK2*, and *AK7* genes are associated with severe human diseases (14, 47, 78, 96). In regulation of cell bioenergetics, adenylate kinase and other phosphotransfer enzyme-governed adenine nucleotide signals provide direct and strictly stoichiometric accounting of energy utilization and transformation balance (36, 41). Another regulator of cell energy metabolism is intracellular Ca^{2+} , which surges during stress, activates dehydrogenases, and primes the energetic system for anticipated energy usage increase (2, 106, 107, 115). Previously we determined metabolic profiles of AK1-deficient muscles and the response to ischemia-reperfusion challenge (65, 99, 102). Here, using ^{18}O -labeling and phosphometabolomic technologies we aimed to establish the high- Ca^{2+} -induced stress response on metabolomic phenotype, phosphometabolite, and phosphotransfer dynamics in hearts of AK1 knockout mice.

At baseline, in the hearts with a null mutation of the *AK1* gene, which encodes the major adenylate kinase isoform, the total adenylate kinase activity and ATP/ADP β -phosphoryl transfer were reduced by 94 and 36%, respectively. The absence of AK1 disrupted the synchrony between P_i turnover at ATP-consuming sites and $\gamma\text{-ATP}$ exchange at ATP synthesis sites, as revealed by ^{18}O -assisted ^{31}P NMR (37). This reduced energetic signal communication in the postischemic heart (99). Moreover, *AK1* gene deletion blunted vascular adenylate kinase phosphotransfer, compromised the contractility-coronary

flow relationship, and precipitated inadequate coronary reflow following ischemia-reperfusion (37). This was associated with upregulation of phosphoryl flux through remaining minor adenylate kinase isoforms and the glycolytic phosphotransfer enzyme, 3-phosphoglycerate kinase (102).

To determine the response of AK1-deficient hearts to high- Ca^{2+} -induced stress, the results from GC-MS metabolomic profiling and ^{18}O -labeling rates were transformed into data sets through multivariate analysis. Initially, data were examined with PCA score plot of the first two score vectors (t1-t2) to assess the data homogeneity, groupings, outliers, and trends. Then the PLS-DA was applied for group discrimination and enhanced information. A robust predictive PLS-DA model was produced [$R^2(X) = 0.72$; $R^2(Y) = 0.92$; $Q^2 = 0.67$ for the two components] to model the metabolic changes associated with gene deletion. This model passed cross-validation according to a random 100 permutations of the class matrix, which indicates validity of the method. As seen in Fig. 4A, clear clustering was obtained between two groups. High- Ca^{2+} stress induced a rightward shift in AK1-deficient hearts' metabolomic profile. To determine significant metabolites in the group differentiation VIP and regression coefficient plots were used (Fig. 4, B and C). Levels of ATP, G-6-P, fructose, adenosine, G-3-P, ADP, alanine, succinate, lactate, and sucrose and turnover rates of $\text{CrP}[^{18}\text{O}]$, G-1-P $[^{18}\text{O}]$, urea $[^{18}\text{O}]$, and $\gamma\text{-ATP}[^{18}\text{O}]$ were included in signature metabolomic profile of high- Ca^{2+} response of AK1-deficient hearts. A surprising finding was the increase in urea ^{18}O -labeling, which reflects accelerated mitochondria-cytosolic urea cycle turnover in stressed hearts. This finding, along with previous metabolomic data on urea cycle metabolites, adds an additional discriminative capability for metabolic stress and coronary artery diseases compared with models containing only clinical variables (111). The association between urea cycle metabolites and prevalence of coronary artery diseases may reflect increased amino acid and ammonia catabolism (82). Thus, beside phosphometabolite turnover, ^{18}O methodology can measure dynamics and provide valuable information regarding changes in activity of other important metabolic processes.

Genetic deletion of AK1 removed all but 6% of total myocardial adenylate kinase activity, yet the intracellular adenylate kinase phosphotransfer flux was only halved in AK1 knockout hearts, indicating an increased functional load on remaining adenylate kinase isoforms. At baseline, reduced adenylate kinase-catalyzed phosphotransfer induced rearrangements in adenine nucleotide and glycolytic metabolism, shifting cellular energetics into an apparently new steady state (37, 99, 102). Here, high- Ca^{2+} -induced stress produced a differential metabolomic profile of the AK1 $^{-/-}$ knockout mice hearts as seen in the PLS-DA score plot (Fig. 4A). To determine significant metabolites in the group differentiation VIP and regression coefficient plots were compared (Fig. 4, B and C). Higher G-6-P and lactic acid levels in the high- Ca^{2+} group indicate increased glycolytic metabolism, while higher adenosine, ADP, and ATP levels suggest increased nucleotide metabolism (Fig. 4D). Ca^{2+} is a known activator of mitochondrial dehydrogenases and a primer of increased ATP production, which is reflected here in higher CrP level and $\text{CrP}[^{18}\text{O}]$ turnover (Fig. 4C) (53, 115). Increased G-3-P levels with almost no changes in G-3-P $[^{18}\text{O}]$ turnover do not allow us to make conclusions on metabolic activity of the substrate shuttle.

Increased succinic acid and β -alanine and decreased aspartate levels indicate altered Krebs cycle and transamination activities. Diminished G-1-P $[^{18}\text{O}]$ turnover indicates reduced glycogen synthesis during myocardial stress. From these plots, it is concluded that glycolytic and glycogenolytic pathways, Krebs and urea cycles, phosphotransfer pathways, and nucleotide metabolism have been altered significantly in AK1 knockouts (Fig. 4D). Based on previous studies (37, 99, 102) and from results presented here we can conclude that AK $^{-/-}$ hearts are more vulnerable to metabolic stress induced by high Ca^{2+} compared with wild type. A detailed comparison of differences in myocardial and plasma metabolomic response between AK1 $^{-/-}$ and wild-type hearts to functional load will be presented in a following separate publication. Taken together, these metabolic changes indicate altered system-wide stress response of cellular energy metabolism after deletion of one significant node in the network.

Dynamic Metabolomic Profile of CK M-CK/CKmit-deficient Hearts

The versatility of stable isotope ^{18}O -assisted ^{31}P NMR and MS in determining dynamic energetic phenotypes of transgenic animals is further highlighted by studies of CK (M-CK/ScCKmit $^{-/-}$)-deficient mice (38) (Fig. 5). CK-catalyzed phosphotransfer is the major component of energy transfer and distribution network in the heart, and compromised CK function is a hallmark of abnormal bioenergetics in diseased hearts (38, 63, 106). A pair-wise PLS-DA analysis of wild-type and M-CK/ScCKmit $^{-/-}$ hearts (Fig. 5, A and B) allowed comparison of metabolic variables important in phenotype determination and group separation. The PLS-DA score plot shows a clear separation between metabolomic profiles of the groups, while the VIP plot of the PLS-DA method indicates the importance of metabolites and turnover rates in discriminating between metabolomic profiles of the groups. Regression coefficient plots of metabolic variables in the PLS-DA model (Fig. 5C) specify metabolic alterations where larger coefficient values (positive or negative) indicate a stronger correlation with group metabolic profile classification. The metabolomic profile of M-CK/ScCKmit $^{-/-}$ hearts is characterized by the higher G-6-P turnover rate, G-6-P level, glycolytic capacity, γ/β -phosphoryl of GTP turnover, as well as β -ATP and β -ADP turnover, indicating altered glycolytic and adenylate kinase metabolic flux. Increased turnover of γ/β -phosphoryls of GTP reflects augmented metabolic flux through NDPK, the Krebs cycle enzyme succinyl CoA synthase, and NMPK in metabolically adapted M-CK/ScCKmit $^{-/-}$ hearts (Fig. 5D). The values of $R^2 = 0.69$ and $Q^2 = 0.95$ indicate the validity of the method and that the model was stable and good to fitness and prediction (Fig. 5E). Thus, multivariate analysis revealed a unique metabolomic signature for M-CK/ScCKmit $^{-/-}$ hearts, suggesting that rearrangements in phosphotransfer and substrate utilization networks provide compensation to genetic CK deficiency.

These results are consistent with previous studies of CK-deficient hearts by others. Increased activities of glycolytic enzymes such as pyruvate kinase and GAPDH were also found in hearts of CK knockout animals (18, 119). M-CK-deficient cardiomyocytes display a higher sensitivity to glycolytic inhibition manifested in premature opening of ATP-sensitive po-

Dynamic metabolomic profiling of AK1-/- hearts

Fig. 4. Phosphometabolomic profiling of the response of adenylate kinase AK1^{-/-} mutant hearts to metabolic stress induced by high Ca^{2+} . **A**: partial least squares discriminant analysis (PLS-DA) score plot of metabolomic profiles of perfused hearts shows clear separation between low (Ca^{2+}) (AK1L) and high- Ca^{2+} (AK1H) groups. **B**: variable importance in the projection (VIP) plot of the PLS-DA method represents a biomarker panel arranged according to the importance of metabolites in discriminating between metabolomic profiles of the groups. **C**: regression coefficient plots of metabolic variables in the PLS-DA model indicating most important metabolite changes (increases or decreases) in high- Ca^{2+} compared with low- Ca^{2+} AK1-deficient hearts; larger coefficient values (positive or negative) indicate a stronger correlation with group metabolic profile classification. **D**: metabolic pathways specifically affected by high Ca^{2+} in AK1^{-/-} hearts. **E**: validation of the PLS-DA model by comparison to the classification statistics of models generated after (100) random permutations of the class matrix; $n = 5$ in each group.

Dynamic metabolomic profiling of M-CK/CKmit^{-/-} hearts

Fig. 5. Dynamic metabolomic profiling of creatine kinase M-CK/CKmit^{-/-} mutant hearts. **A**: PLS-DA of metabolomic profiles of wild-type (WT) vs. M-CK/ScCKmit^{-/-} mutant hearts indicates different metabolic phenotypes. **B**: VIP plot of the PLS-DA method represents dynamic biomarker panel arranged according to the importance of metabolites and turnover rates in discriminating between metabolomic profiles of the groups. **C**: regression coefficient plots of metabolic variables in the PLS-DA model indicating most important metabolic changes (increases or decreases) in M-CK/ScCKmit^{-/-} hearts compared with WT hearts; larger coefficient values (positive or negative) indicate a stronger correlation with group metabolic profile classification. **D**: metabolic pathways specifically affected M-CK/ScCKmit^{-/-} hearts compared with WT hearts. **E**: validation of the PLS-DA model by comparison to the classification statistics of models generated after (100) random permutations of the class matrix; $n = 6-7$ in each group.

tassium channels and shortening of action potential compared with wild type (1), suggesting a greater reliance on glycolytic metabolism. Although hearts deficient in the major CK isoforms have no gross basal functional abnormalities, under an increased load they cannot sustain normal global ATP/ADP ratios, indicating a compromised communication between ATP-consuming and ATP-generating cellular sites (38, 109). This renders contractions to be more energetically costly, forcing the heart to operate under less efficient cardiac bioenergetics (65, 108). Such energetic abnormalities reduce the ability of the myocardium to respond to β -adrenergic stimulation (29), and CK-deficient hearts are more vulnerable to ischemia-reperfusion injury (113). To this end, compensation provided by adenylate kinase and glycolytic phosphotransfers in CK-deficient muscles indicate their integral role in facilitating intracellular high-energy phosphoryl exchange especially under the conditions of genetic or metabolic stress. Thus, the dynamic phosphometabolomic profiling of adenylate kinase and CK-deficient hearts revealed rearrangements and adaptations in the heart energetic system with an induced shift in glycolytic, guanine nucleotide, and CK phosphotransfer pathways and substrate utilization networks.

Conclusions

In the current postgenomic era there is an increasing need for an integrative and systemic view of the molecular regulation of cells and whole organisms. Dealing only with a large number of parameters, data sets, or abstract mathematical network models, such as in bioinformatics, doesn't constitute a viable system approach. An integrative view necessitates positioning data in space and time within real biological networks and modules. The emerging system and network perspective of cardiac metabolism, energetic system, and metabolic sensing circuits has revolutionized the understanding of how normal heart performance is integrated and synchronized with mitochondrial energy transformation and heart electrical activity and how dyssynchronies develop in failing hearts and during atrial fibrillation (1, 22, 24, 35, 39, 41, 42, 106, 124). Accumulation of multiple defects in metabolic networks and exhaustion of compensatory mechanisms leading to the systemic failure of mechano-energetic coupling is a key feature of failing hearts (39, 63). The emergence of metabolomics, a new "omic" science, offers new perspectives in biological regulation, metabolic phenotyping, metabolic monitoring, as well as in elucidation of molecular mechanism and biomarker research of different human diseases (11, 33, 50, 69, 81). While transcriptomics and proteomics data indicate what changes might happen, metabolomic analyses reflect the real status and dynamics of metabolic pathways at a given time point or in a particular physiological or disease state (81). Wide-range metabolomic screening is especially important for determining inborn errors of metabolism, of target drug toxicity, human metabolic phenotypes, and predisposition to specific diseases (69). Importantly, the pharmacometabolomic analysis of metabolic phenotypes can predict individual variation in the pharmacokinetics of drugs without the need for genetic fingerprinting (98). In particular, metabolomic profiling and flux analysis are necessary to reveal the plasticity and restructuring of cellular bioenergetic systems in response to genetic deficiencies and disease conditions (65, 66, 77, 94, 97, 100). Also metabolomic profiling of hearts utilizing different substrates or a cocktail of substrates is warranted since

under physiological conditions hearts have metabolic flexibility and can switch from one substrate to another depending on nutritional and functional states (85).

The emerging ^{18}O -assisted ^{31}P NMR and MS technique provides a versatile methodology allowing simultaneous recordings of multiple parameters of cellular bioenergetics and characterization of metabolic fluxes through different energetic pathways (101, 133, 134). This includes simultaneous recordings of ATP synthesis and utilization, phosphotransfer fluxes through adenylate kinase, CK, and glycolytic pathways, as well as mitochondrial Krebs cycle and associated nucleotide turnover, urea cycle, and glycogen metabolism. This methodology has also a unique capability to measure intracellular energetic communication by comparing kinetics of P_i ^{18}O -labeling (in ATPase compartment) to that of γ -ATP (in ATP synthesis compartment), which is disrupted in ischemia-reperfusion and heart genetic deficiencies (37, 57, 101). Integrated kinetic data obtained using ^{18}O -labeling technology have provided a basis for development of the cardiac system bioenergetics concept where major ATP-consuming and ATP-generating processes are interconnected by phosphotransfer networks composed by adenylate kinase and CK circuits and glycolytic/glycogenolytic network nodes (35, 41). Further development and validation of this advanced methodology specifically designed for profiling of tissue and cellular G-3-[^{18}O]P turnover and mitochondrial substrate shuttle dynamics can be rapidly applied in translational research for diagnosis of substrate shuttle, mitochondrial energetic, and lipid metabolic deficiencies such as in sudden death syndrome (118). Here, metabolomic and fluxomic profiling of atrial myocardium and phosphotransfer enzyme-deficient transgenic animals (AK1 $^{-/-}$ and M-CK/ScCKmit $^{-/-}$) using ^{18}O -assisted GC-MS and ^{31}P NMR indicates metabolic perturbations, flux redistribution, and adaptations in the whole energetic system.

^{18}O -labeling methodology allows quantitative evaluation of cellular ATP turnover and the distribution of energy fluxes between different phosphotransfer pathways in vivo (38). In perfused rat hearts, CK flux increases linearly with cardiac performance, reaching a value close to $330 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein with the heart performing at $\sim 35,000 \text{ mmHg} \cdot \text{min}$, which corresponds to PCr/ O_2 ratio close to 5 (101, 106). This indicates that the CK pathway processes the majority of high-energy phosphoryl flux from mitochondria to ATPases under normal physiological conditions in myocardium (107). About 15–30% of cellular high-energy phosphoryls is carried by the adenylate kinase-glycolytic system, whose contribution increases with muscle contraction and in failing hearts (38, 44, 133). Quantitative phosphotransfer flux distribution and substrate utilization profiling of mouse hearts and skeletal muscles uncovered rearrangements in metabolic phenotype due to genetic deficiencies and intrinsic robustness of energetic system (38, 43, 65, 99).

Previously, the use of metabolite ^{18}O -labeling methodology permitted uncovering fundamental phototransduction and signal transduction events where dynamics but not the levels of cGMP and cAMP correlated with the intensity of light stimuli and signal response (4, 30, 54). Based on these observations it is now clear that simple diffusional exchange of protein-bound nucleotides, as is the case with the transition in G proteins from the GDP- to the GTP-liganded state and also within nucleotide-gated ion channels, is a rather slow process (30, 54); however, in contrast adjacent fast phosphotransfer reactions can assure rapid switching between the

liganded states of nucleotide-gated proteins, determining a fast signal on-off response (42, 45, 60). This provides an explanation of why the response of metabolic sensors such as K-ATP and AMPK doesn't always follow bulk nucleotide levels (35, 36). Further ^{18}O -labeling studies have revealed the significance of energetic dynamics in regulating muscle contraction, metabolic sensing, and insulin secretion, as well as defects in heart failure and atrial fibrillation (24, 43, 44, 46, 95, 133–135). Based on these studies the concept of near-equilibrium enzymatic flux transfer chains operating by ligand conduction mechanism was developed (35, 41, 45). Such a mechanism could provide the means for moving ATP and other metabolites around the cell at a rate faster than simple diffusion (41, 45). Stable isotope ^{18}O labeling technique was indispensable for the metabolic characterization of transgenic animals with deleted *AK1*, *M-CK*, *M-CK/AK1*, *M-CK/CKmit*, and K-ATP channel subunit *Kir6.2* genes (1, 22, 37, 57, 65, 66, 99, 102). Studies of AK1, M-CK, M-CK/AK1, and M-CK/CKmit knockout mice revealed an inherent plasticity of the cellular energetic system and redistribution of phosphotransfer flux between parallel chains, thus alleviating energetic hindrances caused by genetic deficiency (38, 43, 64, 65, 99). Recently, by use of a novel superfast magnetization saturation transfer method it was demonstrated that in the in vivo normal heart, redundant multiple supporting systems of myocardial ATP production, transfer, and utilization exist such that inhibition of one pathway does not compromise contractile performance acutely (129). Labeling studies with the ^{18}O also provided direct evidence for the critical role of adenylate kinase, CK, and glycolytic phosphotransfers in transmitting energetic signals to membrane metabolic sensors K-ATP channels (1, 22). Metabolic interrogation using the ^{18}O -labeling technique of hearts from K-ATP channel-deficient mice, which have compromised metabolic sensing, stress response, and hormonal imbalances (70, 88, 89), revealed energetic deficits and metabolic rearrangements consistent with altered calcium and hormonal regulation of cellular energy metabolism (56, 57). In this regard, previous in vivo studies indicate that increased stress and myocardial work during dobutamine infusion in large animal hearts results in significant increases of blood flow and MVO_2 , with the decrease in Pcr/ATP ratio reflecting changes in the kinetics of oxidative phosphorylation or intermediary metabolic steps (90). The metabolomic and ^{18}O -labeling profiling presented here allows further delineation of subtle changes in multiple energetic and metabolic reactions under functional stress.

In summary, phosphometabolomics technologies provide a previously unavailable tool for the dynamic analysis of perturbations in metabolic networks induced by diseases or genetic and acquired metabolic deficiencies. This stable isotope ^{18}O -labeling technique is capable of monitoring phosphotransfer reactions and energetic dynamics in all systems of interest in living tissues. It provides a suitable platform for physiological genomic studies and for prediction and diagnosis of human metabolic diseases states. Our studies demonstrate that this approach is valuable for metabolomic and fluxomic profiling of preconditioned and failing human hearts, as well as for metabolic phenotyping of transgenic animal models simulating human diseases, and for establishing biomarkers and diagnosis of mitochondrial energetic deficiencies (43, 65, 99–102). Thus, emerging new methodologies for metabolomic analysis, in conjunction with system and network approaches, provide new avenues for better understanding of cellular energetic system in health and diseases.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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