Genetic Profiling Reveals Global Changes in Multiple Biological Pathways in the Hearts of Na, K-ATPase Alpha 1 Isoform Haploinsufficient Mice

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Key Words

Sodium pump • Na,K-ATPase • Microarray • Cardiac • Energy metabolism

Abstract

The Na,K-ATPase transports three sodium ions out of the cell and two potassium ions into the cell using ATP hydrolysis for energy. The ion gradient formed by the Na,K-ATPase contributes to the resting membrane potential, maintains cellular excitability and is important for glucose and amino acid uptake in the cell. The $\alpha 1$ catalytic isoform is expressed in virtually all cell types. We have previously examined cardiac physiology of mice lacking one copy of the $\alpha 1$ isoform gene of the Na,K-ATPase. The observation of reduced cardiac contractility in the $\alpha 1$ heterozygous mice was unexpected since mice heterozygous for the $\alpha 2$ isoform displayed enhanced cardiac contractility similar to what would be observed with cardiac glycoside treatment. We further examined hearts from $\alpha 1$ heterozygous mice to identify genomic responses to reduced Na,K-ATPase capacity. Using microarray analyses, we identified groups of genes whose expressions were perturbed in the $\alpha 1$ heterozygous hearts compared to wild-type. Known functional

relationships of these genes suggest that multiple biological pathways are altered by $\alpha 1$ hemizyosity including activation of the renin-angiotensin system, changes in genes of energy metabolism and transport and elevated brain natriuretic peptide. This suggests that Na,K-ATPase $\alpha 1$ isoform activity may be required in numerous cellular processes.

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Introduction

The Na,K-ATPase is a plasma membrane protein composed of α and β subunits that transports sodium and potassium ions against their concentration gradient using ATP for energy. The α -subunit contains the catalytic activity and multiple isoforms exist displaying tissue-specific expression. The exception is the $\alpha 1$ isoform, which is expressed in virtually all tissues.

The importance of the Na,K-ATPase in cardiac muscle is well documented. It contributes to the resting membrane potential and is also important for cardiac contractility [1, 2]. It is well known that cardiac glycosides (digitalis or ouabain) elicit an increase in cardiac

contractility (positive inotropic response) by binding to and inhibiting the Na,K-ATPase. The mechanism of action of digitalis has been proposed to be through a secondary inhibition of the Na/Ca exchange, resulting in elevated intracellular calcium and enhanced force of contraction. It is not known, however, if each of the multiple α isoforms produces the same positive inotropic effect when inhibited by digitalis. To more accurately determine the relative contribution of each Na,K-ATPase isoform to cardiac contractility, genetically engineered mice were produced expressing reduced $\alpha 1$ isoform or reduced $\alpha 2$ isoform. Cardiac contractility experiments were performed using the working heart model and, to our surprise, the $\alpha 1$ heterozygous heart ($\alpha 1^{+/-}$) exhibited reduced contractility, in contrast to the $\alpha 2$ heterozygous heart ($\alpha 2^{+/-}$) whose phenotype mimicked that of digitalis action displaying enhanced contractility along with elevated intracellular calcium [3]. These results suggested that the two Na,K-ATPase isoforms in the mouse heart play different physiological roles. Several studies have supported a theory in which the $\alpha 2$ isoform directly regulates intracellular calcium. It is co-localized with and functionally coupled to the Na/Ca exchanger, thus providing the sodium ion gradient necessary for driving Na/Ca exchanger activity [4-7].

Because the $\alpha 1$ isoform is abundantly expressed in the mouse heart, and its genetic reduction resulted in reduced cardiac performance, we sought to identify the genomic responses to reduced Na,K-ATPase capacity. A gene microarray approach was used to identify alterations in global biological processes in hearts from the $\alpha 1^{+/-}$ mice. We observed changes in gene expression that function in several cellular processes of the heart including energy metabolism, ion transport and altered natriuretic hormone expression. The microarray results demonstrate that the Na,K-ATPase $\alpha 1$ isoform influences the expression of a variety of biological systems and suggest crucial cellular roles for this ion transporter.

Materials and Methods

Preparing poly A⁺ RNA from mouse tissues

Generation of gene targeted mice lacking one allele of the Na,K-ATPase $\alpha 1$ isoform has been described previously [3]. Mice were housed in a barrier facility and handled in accordance with the University of Cincinnati Institutional Animal Care and Use Committee. Experiments were performed on wild-type and $\alpha 1^{+/-}$ mice, 8-16 weeks of age, carrying a Black Swiss/129 genetic background. Immediately after euthanizing the mice, hearts were removed, excess blood blotted away and tissue

homogenized in Tri Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was isolated by the manufacturer's recommended protocol, and was followed by a second round of phenol and chloroform extraction and ethanol precipitation. RNA integrity was verified by glyoxyl gel electrophoresis. Two independent pools of hearts (n=5), using two separate chips for each genotype, were used. We submitted 10 μ g of total RNA at 1 μ g/ μ l per microarray chip to the Affymetrix Core Facility (Children's Hospital Research Foundation, Cincinnati, OH) for reverse transcription and cRNA synthesis. Microarray hybridization was performed on the Affymetrix Mouse Chip U74Av2 using Affymetrix-recommended protocols.

Affymetrix GeneChip Analysis

We used Affymetrix software to analyze the hybridization quality using the parameters of signal to background fluorescence, spot geometry, and an assessment of dynamic range exhibited by each fluorescence channel. The data were analyzed using GeneSpring software version 5.0 (Silicon Genetics, Redwood City, CA) to identify those genes whose expressions were most altered by $\alpha 1$ isoform deficiency. An intensity-dependent normalization procedure was performed to ensure linearity of the signal throughout high and low fluorescent levels. Of the 7,347 genes represented on the chip, we excluded those called "absent" by Affymetrix software in any of the samples. We then performed Student's T-test and selected those genes that were up- or down-regulated having a significance of $p < 0.01$ in the $\alpha 1^{+/-}$ hearts compared to wild-type. From the resulting 770 genes, we further characterized the top-ranked most altered genes as those having a 2-fold change either upregulated or down-regulated in $\alpha 1^{+/-}$ hearts compared to wild-type. This procedure identified 120 genes that were up-regulated and 349 genes that were down-regulated in hearts from the $\alpha 1^{+/-}$ mice. These genes were then grouped according to function: transport (Table 1), metabolism (Table 2), signal transduction (Table 3) and cell proliferation/DNA repair (Table 4). Note that a log ratio value of greater than 1 is regarded as upregulated and a log ratio value of less than 1 is regarded as a down-regulated gene. Other ontology groups include structure/motor, development, apoptosis, immune/inflammation, transcription/translation and cell proliferation/DNA repair. These are not shown here, however the raw microarray data can be found at the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database website. The GEO accession numbers are GSM19023, GSM19024, GSM19025 and GSM19026.

Semi-quantitative RT-PCR

Changes in expression of selected genes were verified by real-time RT-PCR from the same samples used for gene chip analysis. Following treatment with RQ1 DNase (Promega, Madison, WI), 2 μ g of total RNA per gene were reverse transcribed using the SuperScript II reverse transcription kit (Invitrogen, Carlsbad, CA) followed by real-time PCR using SYBR Green I (Molecular Probes, Eugene, OR). Primers were designed from the annotated GeneChip sequences and the identity confirmed by BLAST analysis (National Center for Biotechnology Information, NCBI). Primer sequences used are

Table 1. Micro-array analysis of genes functioning in transport. The average normalized spot intensity of two replicates is given with statistical p-value for both.

Transport	Genbank	Avg Normalized	Avg p-value
Up-regulated			
Myotonic dystrophy protein kinase	Z38015	6.38	1.48E-08
Potassium leak channel, Kcnk3	AF065162	5.97	3.97E-07
Hyperpolarization-activated cation channel,HCN2	NM_008226	2.91	1.78E-06
Wolframin	NM_011716	2.90	2.30E-07
Glypican 1	NM_016696	2.21	2.05E-08
Sodium- and chloride-dependent creatine transporter	NM_133987	2.18	9.92E-08
L-type voltage dependent calcium channel, alpha 1C	NM_009781	2.03	6.06E-08
HDL-binding protein	BC027788	1.99	1.93E-08
Down-regulated			
ATP-binding cassette transporter-G1	NM_009593	0.45	4.87E-06
Nucleotide binding protein-1	NM_011955	0.43	1.30E-04
ATP-binding cassette transporter-A1	NM_013454	0.43	1.03E-03
ATP-binding cassette transporter-B2	AK040919	0.36	7.02E-06
Calcium activated potassium channel-N4	AK038253	0.34	5.41E-05
Calcium ATPase, SERCA -1	BC036292	0.18	1.89E-06
Voltage-gated potassium channel	NM_008418	0.10	4.75E-04

Table 2. Micro-array analysis of genes functioning in signal transduction. The average normalized spot intensity of two replicates is given with statistical p-value for both upregulated and downregulated genes.

Signal Transduction	Genbank	Avg Normalized	Avg p-value
Up-regulated			
Peptidylglycine alpha-amidating monooxygenase	NM_013626	3.38	1.38E-08
Brain natriuretic peptide	D16497	3.12	2.10E-08
Receptor activity modifying protein 2	NM_019444	2.87	1.02E-07
Guanine nucleotide binding protein alpha 12	NM_010302	2.56	5.19E-08
Bone morphogenetic protein 6	NM_007556	2.15	2.88E-05
S100 calcium binding protein a1	NM_011309	2.09	1.02E-08
Src homology 2 domain-containing transforming protein C1	BC036172	2.05	3.76E-07
Lipid phosphate phosphatase 1	NM_008903	1.79	4.65E-07
Crystallin alpha C	NM_030704	1.75	2.44E-04
Ras-like, family 2, locus 9	NM_009028	1.65	4.40E-05
Interferon-inducible GTPase	NM_021792	1.20	7.83E-07
Down-regulated			
Multiple inositol polyphosphate histidine phosphatase 1	NM_010799	0.43	3.63E-07
Regulator of G-protein signaling-19	NM_026446	0.42	7.42E-05
Brain acid soluble protein 1	AK046868	0.40	4.94E-06
Gamma interferon-induced GTPase	NM_018738	0.37	8.78E-06
Adenylyl cyclase associated protein	NM_007598	0.37	2.92E-05
Hematopoietic cell specific Lyn substrate 1	NM_008225	0.36	3.90E-04
Protein tyrosine kinase 2B	NM_172498	0.33	2.47E-05
ADP-ribosylation-like factor 6 interacting protein 2	BC006934	0.32	1.25E-07
Protein tyrosine phosphatase non-receptor type 18	NM_011206	0.31	9.77E-05
Regulator of G-protein signaling-14	NM_016758	0.28	1.50E-05
Regulator of G-protein signaling-10	NM_026418	0.25	3.72E-06
Src homology domain interacting protein-2	NM_019436	0.24	4.55E-05
Ermelin	NM_139143	0.23	6.32E-05
MARCKS-like protein	NM_010807	0.23	2.37E-06
Rho GTPase Rac2	NM_009008	0.22	3.46E-06
Calcitonin receptor modifying protein, Ramp-1	NM_016894	0.21	1.03E-06
Protein tyrosine phosphatase SHP-1	NM_013545	0.19	1.76E-04
Protein kinase C beta	NM_008855	0.14	1.16E-06
GABA-B receptor	AL078630	0.11	9.89E-04
Src like adaptor protein	AK036167	0.05	3.34E-03

Table 3. Microarray analysis of genes functioning in energy metabolism. The average normalized spot intensity of two replicates is given with statistical p-value for both upregulated and downregulated genes.

Energy Metabolism	Genbank	Avg Normalized	Avg p-value
Up-regulated			
Pyruvate Dehydrogenase Kinase, isoenzyme 4	NM_013743	3.75	1.32E-08
Thrombomodulin	NM_009378	3.05	1.51E-07
Uncoupling protein 3	AB010742	2.76	8.98E-07
Plasma membrane-associated protein S3-12	NM_020568	2.70	1.05E-07
Aldehyde oxidase 1	NM_009676	2.70	2.74E-06
Oxoglutarate dehydrogenase (lipoamide)	BC013670	2.57	6.82E-09
Adenylate kinase 1	NM_021515	2.56	1.31E-08
Sorbin 1	NM_009166	2.38	2.30E-08
Glycogen phosphorylase	NM_011224	2.30	1.32E-08
Succinyl CoA:3 oxo acid CoA transferase	NM_024188	2.28	1.24E-08
Angiotensin converting enzyme	BC040404	2.24	2.54E-04
Adenylate kinase 4	NM_009647	2.23	3.94E-07
Aldehyde dehydrogenase 1	NM_013467	2.13	2.89E-08
Branched chain ketoacid dehydrogenase E1	NM_007533	2.08	4.80E-08
Ceruloplasmin	NM_007752	2.08	9.26E-08
Thioether S-methyltransferase	NM_009349	2.08	5.28E-07
Catechol-O-methyltransferase	NM_007744	2.07	2.52E-08
Alpha-methylacyl-CoA racemase	AK048249	2.04	4.44E-05
ART3	Y08027	1.99	2.98E-08
Creatine kinase, muscle	NM_007710	1.87	1.84E-07
Amylo-1, 6-glucosidase, 4-alpha-glucotransferase	AK020402	1.56	4.20E-07
Down-regulated			
Laminin receptor 1	NM_011029	0.44	5.09E-07
Serine hydroxymethyl transferase 1	BC026055	0.44	3.22E-04
Resistin	NM_022984	0.41	3.46E-04
UDP-glucose ceramide glucosyltransferase	BG921641	0.41	3.27E-07
Isopentenyl-diphosphate delta isomerase	NM_145360	0.35	1.07E-04
Long chain fatty acyl elongase	NM_130450	0.34	8.03E-04
Citrate lyase	AF332052	0.34	9.97E-07
Transketolase	AK030446	0.32	5.80E-08
Atp synthase	NM_020615	0.30	1.39E-07
Glucose-6-phosphate dehydrogenase	Z84471	0.30	4.01E-04
Phosphatidylinositol-4-phosphate 5-kinase	AK012196	0.28	1.86E-07
Aryl hydrocarbon receptor nuclear translocator	NM_007489	0.27	5.56E-05
Stearoyl-Coenzyme A desaturase 2	NM_009128	0.24	2.97E-07
Methylenetetrahydrofolate dehydrogenase-cyclohydrolase	NM_008638	0.22	1.67E-04
Phosphoserine aminotransferase	BC004827	0.17	1.47E-05
Adenosine deaminase	M14167	0.17	1.99E-04

listed in Table 5. PCR primer quality was tested by melting curve analysis. Relative quantification based on standard curves was calculated using the efficiency calibration described [8]. For FXYD1, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. For all other genes, 18S rRNA was used as an internal control. The internal controls were verified to ensure that they did not change expression in $\alpha 1^{+/-}$ hearts compared to wild-type. The PCR primer sequences for the 18S control (accession # X00686) are 5'AGTCCCTGCCCTTTGTACACA3' for the upstream primer and 5'CCGAGGGCCTCACTAAACC3' for the downstream primer. The PCR primer sequence for the GAPDH control (accession # NM008084) are 5'CCAGTGAGCTTCCCGTTCA3' for the upstream primer and 5'GAACATCATCCCTGC-

ATCCA3' for the downstream primer. Table 5 includes a list of primer sequences along with the quantitative value for each gene analyzed using real-time PCR. It is important to note that while the absolute values for expression by microarray using log ratio are not identical to the fold-change by RT-PCR, the general trend regarding increased or decreased expression are the same in virtually all genes, with the exception of ANP (Table 5).

Blood analysis of aldosterone

The changes in gene expression observed from the microarray analysis, specifically angiotensin converting enzyme (Table 3) suggested that the renin-angiotensin system may be activated in the $\alpha 1^{+/-}$ mice. Concentrations of aldosterone in

Table 4. Microarray analysis of genes functioning in cell proliferation and DNA repair. The average normalized spot intensity of two replicates is given with statistical p-value for both upregulated and downregulated genes. The downregulated gene list shown is the 15 genes most highly downregulated from a total of 61 genes (see Supplemental Table 1).

Cell proliferation/DNA repair	Genbank	Avg Normalized	Avg p-Value
Up-regulated			
Upregulated during skeletal muscle growth 4	NM_031401	2.89	9.72E-09
Connective tissue growth factor	NM_010217	2.67	4.38E-08
Glutathione peroxidase 3	NM_008161	2.41	6.15E-09
Vascular endothelial growth factor A	NM_009505	2.41	1.97E-07
Mitogen-activated protein kinase 12	NM_013871	2.27	2.99E-07
Vascular endothelial growth factor receptor, Flt1	NM_010228	2.24	1.56E-06
Neural proliferation, differentiation and control gene-1	NM_008721	2.04	1.76E-05
Midline 2	NM_011845	2.04	3.00E-04
Neurturin	NM_008738	2.03	6.04E-07
Cystein-rich protein 61	NM_010516	1.78	2.95E-05
Mu 5 class Glutathione S-transferase	NM_010360	1.65	4.70E-08
Acetylhydrolase 2	NM_133880	1.26	1.15E-03
Down-regulated			
Histone H2AX	Z35401	0.18	1.62E-07
Stathmin 1	NM_019641	0.18	2.13E-07
Pax1 transcription factor	M20978	0.18	3.41E-04
Topoisomerase II alpha	NM_011623	0.17	3.39E-04
Flap endonuclease 1	NM_007999	0.15	5.63E-06
Mini chromosome maintenance deficient protein	AK088142	0.15	2.46E-05
Serine/threonine kinase 6	BC014711	0.15	1.11E-03
Proliferating cell nuclear antigen	X57800	0.15	5.21E-08
Serine/threonine kinase 18	NM_011495	0.14	2.48E-06
Smardc2	NM_031878	0.14	1.29E-07
Cell division cycle 6 homolog	NM_011799	0.12	1.48E-04
Ect2	NM_007900	0.08	2.39E-05
Ribonucleotide reductase M2	NM_009104	0.08	6.86E-06
Nuclear protein 95	NM_010931	0.08	3.89E-04
Cyclin B1	NM_007629	0.05	6.61E-04

serum were determined using a ^{125}I radioimmunoassay, performed according to the manufacturer's suggested protocol (Diagnostics Products, Los Angeles, CA). Values are reported as the average ($\pm$ SEM) from 4 wild-type animals and 8 $\alpha 1^{+/-}$ animals performed in triplicate.

Cardiovascular performance in intact closed chest animals

Previous examination of cardiac performance of the $\alpha 1^{+/-}$ mice was performed *ex vivo* in the absence of neurohormonal influence using the Work Performing Heart method [3]. We performed additional experiments in this report to determine cardiac function *in vivo*. We measured cardiac function both at baseline and following dobutamine infusion to increase the work-load of the heart. Adult male mice were anesthetized with an intraperitoneal injection of 50 μg ketamine/g of body weight and 100 μg thiobutabarbital/g of body weight (Inactin, Research Biochemical International, Natick, MA). Procedures for measuring the mean arterial pressure, intraventricular pressure, heart rate and maximum and minimum dP/dt have been described previously [7]. Average values for heart rate, mean arterial pressure, and systolic left ventricular pressure were measured from the pressure waveforms and determined for each animal

from at least 50 consecutive beats during the final 30 s of each 3-min dosage period. Maximum dP/dt for each dobutamine infusion was calculated from the first derivative of the pressure waveforms.

Statistical analysis

Results are expressed as means $\pm$ SE. Significance was estimated by Student's t-test for paired observations, $P \leq 0.01$ was considered significant for microarray analyses and $P \leq 0.05$ was considered significant for aldosterone quantitation. For the *in vivo* cardiovascular performance analysis, a two-factor analysis of variance was used with repeated measure on the second factor, statistical significance was $P < 0.05$.

Results and Discussion

Identification of general trends in gene expression in $\alpha 1^{+/-}$ hearts

Microarray analysis, used to examine global changes in gene expression in hearts from $\alpha 1^{+/-}$ mice, identified a large number of genes altered in expression compared to

Table 5. Validation of mRNA abundance by microarray analysis using real-time PCR. FXYD1, phospholemman; MDPK, myotonic dystrophy protein kinase; ACE, angiotensin converting enzyme; G6PDH, glucose-6-phosphate dehydrogenase; KcnK3, potassium leak channel; BNP, brain natriuretic peptide; PDHK4, pyruvate dehydrogenase kinase-4; RXR, retinoid X receptor; PAM, peptidylglycine alpha-amidating monooxygenase; ANP, atrial natriuretic peptide; PPAR alpha, peroxisome proliferators-activated receptor alpha. For FXYD1, GAPDH (glyceraldehyde phosphate dehydrogenase) was used as an internal control and for all other genes the 18S rRNA was used as an internal control. n/a, gene not represented on microarray chip.

Gene	GenBank#	Forward Primer	Reverse Primer	Fold-change, PCR	Fold-change, array
FXYD1	AF091390	5'CGGGAAATA AGGCCTGCATTA3'	5'TCGGGTTCCC CAGTTCTAAAA3'	3.74	2.1
MDPK	Z38015	5'GTTCCGTATT TAGGTAGCGGTGG3'	5'CCGGAAGCAC GACACCTG3'	1.58	6.4
ACE	BC040404	5'GCCTTTCCCA GACAAACCC3'	5'AGCATTGACT CCGCCAG3'	1.97	2.24
ATP Synthase	NM020615	5'GGAGTACAA TCTGGCCAACCTC3'	5'CGTCTTGCTG GCGTTGTC3'	0.45	0.30
G6PDH	Z84471	5'CATGGTGCT GAGGTTTGCTAAC3'	5'GTAGCAGGCT TTTCCATGGC3'	0.22	0.29
KcnK3	AF065162	5'CATCGGCTAT GGTCATGCG3'	5'CTGGAACATG ACTAGTGTGAGCG3'	3.04	5.97
Creatine kinase	NM_007710	5'GCAAGCACA ACAATCACATGG3'	5'GATGACATCG TCCAGAGTGAAGC3'	2.62	1.87
BNP	D16497	5'GCCCAACCC ACTTTACAGGA3'	5'CACCACACCC CCATCTTCAG3'	3.28	3.1
PDHK4	NM_031743	5'GGTAAGGAT CAGCGGGAGG3'	5'CAGCCTTCTG GTTCTTTCTTG3'	2.82	3.75
RXR	NM_009107	5'GCCATCGTG CTGTTTAACCC3'	5'GCCTGGCTGT TCCGGATAC3'	2.28	2.4
PAM	NM_013626	5'CTGGTGAAAG GTCCTTGGGAA3'	5'GGTTATTCTTA GAATCCAGGGCC3'	1.98	3.38
ANP	K02781	5'CGCAGGCCC TGAGTGTGAGCAG3'	5'GCGCCTAGAA GCACTGCCG3'	13.5	1.3
PPAR alpha	NM_011144	5'GGGCTTCTTT CGGCGAAC3'	5'CCGACAGACA GGCACTTG TG3'	6.98	n/a
ATP citrate lyase	AF332052	5'GCCCCAGT GAACAACAGAC3'	5'CTCTCACAAAT GCCCTTGAAGG3'	0.81	0.34

wild-type. A list of the complete set of the Affymetrix data results can be found at the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database website. The GEO accession numbers are GSM19023, GSM19024, GSM19025 and GSM19026. A global summary of the relative number of genes is illustrated in Figure 1 showing that 112 genes were upregulated and 328 genes were down-regulated ($p < 0.01$). The replicates for wild-type chips showed a few genes slightly upregulated or down-regulated as indicated by the blue lines on Figure 1. The two wild-type chips are not equal to unity because one cannot completely exclude slight variations due to technical handling of the samples, as well as variations in gene expression from animal to animal. The genes were then grouped according to function using the following categories: metabolism, structure/motor, development, signal transduction, apoptosis, immune/inflammation, transcription/translation,

transport, cell cycle/DNA repair and unknown genes/EST's. Some general observations regarding trends in gene expression in the $\alpha 1^{+/-}$ hearts were noted. First, of the 328 genes down-regulated, 102 genes functioned in immune/inflammation, 62 genes were cell proliferation/DNA repair genes and 38 genes functioned in transcription/translation. The large number of genes down-regulated in immune/inflammation was unexpected. It is possible that immune cells could have been retained in the heart tissue during isolation of the RNA and that the immune response genes observed are those from circulating immune cells. Interestingly, adenosine deaminase was also found to be down-regulated in the $\alpha 1^{+/-}$ hearts (0.17 log ratio value, Table 2) and lack of this gene is known to cause immunodeficiency [9]. The down-regulation of adenosine deaminase would result in reduced metabolism of adenosine, thereby preserving local adenosine levels. Since adenosine is an important signaling molecule that

Fig. 1. Global changes in cardiac gene expression in Na,K-ATPase $\alpha 1^{+/-}$ versus wild-type adult male mice. Signal intensity is plotted as the log ratio value for each transcript where a value of 1 is equal to that of wild-type, a value less than 1 is decreased expression (blue) and a value greater than 1 is increased expression (orange/red) compared to wild-type. A, one replicate microarray chip and B is a second replicate microarray chip. These genes represent statistically significance changes in expression between $\alpha 1^{+/-}$ and wild-type (WT) ($p < 0.01$) with 112 genes up-regulated and 328 genes downregulated.

regulates blood flow in several organ systems, including heart and kidney, perturbations in adenosine deaminase might be predicted to have an important influence on hemodynamics in these organs. Second, the $\alpha 1^{+/-}$ animals did not display evidence of growth retardation nor small organ size compared to wild-type even though quite a number of cell cycle-related genes were down-regulated. Third, although the Na,K-ATPase is an ion transporter, genetic reduction of the $\alpha 1$ isoform resulted in a surprisingly low number of other ion transporters with altered expression (Table 1). Not unexpectedly, since the Na,K-ATPase contributes to volume regulation, we observed an upregulation of genes that function in cell volume regulation in the $\alpha 1^{+/-}$ mice. Lastly, the Na,K-ATPase uses 20-25% of cellular ATP and one might predict that genetic reduction of the Na,K-ATPase would result in less ATP utilization. However, we observed increases in gene expression of two energy metabolism pathways, fatty acid oxidation and creatine phosphate suggesting that the heart is responding to reduced $\alpha 1$ isoform expression by increasing, not decreasing, energy production.

Because there were a large number of genes altered in expression in the $\alpha 1^{+/-}$ hearts, a detailed analysis of the genes focused on only selected pathways: transport (Table 1), signal transduction (Table 2), energy metabolism (Table 3) and cell proliferation/DNA repair (Table 4). Since the list for down-regulated genes involved in cell proliferation/DNA repair was long, only the 15 genes most down-

regulated were included in Table 4. Note that the changes in expression between $\alpha 1^{+/-}$ and wild-type are log-ratio values and a log ratio value of 1 would be a gene having equal expression in wild-type and $\alpha 1^{+/-}$ mice. As the log ratio value becomes smaller it reflects more down-regulation of the gene in $\alpha 1^{+/-}$ hearts, and likewise, as the log ratio value becomes larger, this reflects more upregulation of the gene in $\alpha 1^{+/-}$ hearts compared to wild-type.

In vivo cardiac performance of wild-type and $\alpha 1^{+/-}$ hearts

Because we observed changes in several genes important to cardiac performance in the $\alpha 1^{+/-}$ mice, including the pacemaker channel and alterations in neurohormones, we determined whether these genomic responses were affecting cardiac performance *in vivo*. For all analyses, eight mice of each genotype were used and examined under increasing concentrations of dobutamine (1 ng/g body weight/min to 32 ng/g body weight/min) to increase cardiac output. Although there appeared to be differences in heart rate between wild-type and $\alpha 1^{+/-}$ mice with increasing dobutamine (Figure 2A), these differences were not statistically significant. At rest (no dobutamine), the maximum dP/dt was reduced in $\alpha 1^{+/-}$ hearts (6919 ± 417 mmHg/s) compared to wild-type (8649 ± 500 mmHg/s), (Figure 2B, $p < 0.05$) and with increasing dose of dobutamine there was no statistical difference between the two genotypes, except at the

highest doses. The minimum dP/dt was slightly reduced in $\alpha 1^{+/-}$ hearts (-8400 ± 449 mmHg/s) compared to wild-type (-9177 ± 527 mmHg/s) but was not statistically significant (Figure 2C). While both the $\alpha 1^{+/-}$ and the wild-type hearts increased contraction and relaxation rate with increasing dobutamine infusion compared to no

Fig. 2. Dose response to dobutamine in anesthetized Na,K-ATPase $\alpha 1^{+/-}$ and wild-type (WT) mice. Graphs show heart rate, beats per minute (BPM) (A), +dP/dt, mmHg/s (B), -dP/dt, mmHg/s (C) and systolic intraventricular pressure, mmHg (D) determined with a catheter advanced into the left ventricle and mean arterial pressure, mmHg (E) measured in the right femoral artery. Values for each animal were determined from at least 50 consecutive beats during the final 30s of each 3-min. dose. Data are derived from 8 WT and 8 $\alpha 1^{+/-}$ mice. * $p \leq 0.05$ using 2-way ANOVA. A statistically significant group effect to dobutamine dose is evident compared to no dobutamine for both wild-type and $\alpha 1^{+/-}$ mice for each parameter tested. Both mean arterial pressure and systolic intraventricular pressure are significantly higher for control and at every dobutamine dose for wild-type versus $\alpha 1^{+/-}$, $p < 0.05$.

dobutamine ($p < 0.05$ at all concentrations, Figure 2), both genotypes responded in a parallel manner that was not statistically significant between genotypes. An important difference, however, is that the $\alpha 1^{+/-}$ mice were different than wild-type when measuring mean arterial pressure and systolic intraventricular pressure. Both parameters were statistically lower in $\alpha 1^{+/-}$ mice compared to wild-

type at every dobutamine dose (Figure 2D and 2E), $p < 0.05$. Both $\alpha 1^{+/-}$ and wild-type pressures increased with increasing doses of dobutamine in a parallel manner compared to no dobutamine control, $p < 0.05$. Our studies indicate that the maximum dP/dt of the $\alpha 1^{+/-}$ hearts displays mildly depressed contractility at baseline, similar to the work performing heart experiments previously

reported [3]. Upon dobutamine-induced stress, however, performance of the $\alpha 1^{+/-}$ hearts increases to very high levels that were not different from that seen in wild-type, indicating that these hearts have ample contractile reserve. Since the $\alpha 1^{+/-}$ heart is capable of contracting at a level similar to that of wild-type under dobutamine-induced stress, it seems likely that the low blood pressure observed in $\alpha 1^{+/-}$ mice throughout the dose-response range reflects a decrease in peripheral resistance or afterload, rather than a primary defect in contractile performance.

Altered transcriptional response of ion transport genes in $\alpha 1^{+/-}$ hearts

The microarray results presented here suggest that reduction of the Na,K-ATPase $\alpha 1$ isoform affects multiple biological pathways in the heart, one of which is ion transport. It has already been reported that the $\alpha 2$ isoform of the Na,K-ATPase is upregulated 50% in the $\alpha 1^{+/-}$ hearts [3], suggesting the heart is attempting to increase Na,K-ATPase content. This led to an examination of the regulatory gamma subunit of the Na,K-ATPase, phospholemman (FYD1), which is known to modulate Na,K-ATPase activity and is highly expressed in heart. While not selected as a significantly altered gene after strict statistical filtering of the microarray data, it is upregulated by 2.1 log ratio (knockout vs wild-type). To verify this upregulation, we used real-time PCR and found it to be 3.74 fold elevated in the $\alpha 1^{+/-}$ hearts (Table 5). Interestingly, Feschenko et al [10] reported that when antibodies against phospholemman are incubated with the Na,K-ATPase, a reduction in enzyme activity was observed. The same group further reported preliminary observations that reduced Na,K-ATPase activity occurs in cardiac tissue in the phospholemman knockout mouse. These data suggest that phospholemman is capable of modulating Na,K-ATPase activity. The hearts from the $\alpha 1^{+/-}$ mice may be attempting to increase Na,K-ATPase activity by at least two different mechanisms: 1) by upregulation of another Na,K-ATPase isoform, the $\alpha 2$ isoform [3] and 2) by upregulation of a modulatory subunit, phospholemman.

Three other genes important to cardiac performance also exhibited significant changes: HCN2, the hyperpolarization-activated cation channel (considered the pacemaker channel), upregulated 2.91 log ratio; Kcnk3, the potassium leak channel (important for the cardiac action potential), upregulated with a 5.97 log ratio; and the L-type calcium channel (important for eliciting calcium-induced calcium release from internal stores

Fig. 3. Elevated serum aldosterone in Na,K-ATPase $\alpha 1^{+/-}$ mice. Blood was removed from adult male wild-type (WT) and $\alpha 1^{+/-}$ mice and assayed for aldosterone using ^{125}I -radioimmunoassay. Results are the mean $\pm$ S.E. of 4 (wild-type) and 8 ($\alpha 1^{+/-}$) animals in triplicate. * $p \leq 0.05$, using Student's t-test.

during depolarization) with a log ratio of 2.03 (Table 1). The potassium ion leak channel, Kcnk3, was the most over-expressed transporter found in the $\alpha 1^{+/-}$ hearts (Tables 1 and 5). Kcnk3 is part of a newly identified potassium channel family that has a non-inactivating background [11]. It has been shown that Kcnk3 mRNA is highly expressed over the entire left ventricle and contributes to the potassium ion flux at the plateau of the cardiac action potential [11]. We expect that a reduction of Na,K-ATPase activity would depolarize the cell and, in order to compensate, the cell might upregulate ion channels such as Kcnk3 to hyperpolarize the cell. Interestingly, the cardiac pacemaker channel, HCN2 was found to be upregulated almost 3-fold (Table 1), but there was no statistical difference in heart rate between $\alpha 1^{+/-}$ and wild-type mice.

Changes in calcium handling genes are an important indication of aberrant cardiac contraction in the $\alpha 1$ hemizygous hearts. It appears that these cells are instigating transcriptional changes to increase cytosolic calcium, which is essential to increasing cardiac contractility. For example, both the voltage dependent L-type Ca^{2+} channel and the EF-hand calcium binding protein S100A are 2-fold elevated in the cell. Both of these genes have been reported to enhance contractility when overexpressed [12, 13], and their increased

Fig. 4. Activation of two opposing cell volume regulatory systems, the renin-angiotensin system and the ANP/BNP natriuretic hormone system in Na,K-ATPase $\alpha 1^{+/-}$ mice. $\uparrow$, upregulation of gene transcription. AT1, angiotensin-1; AT2, angiotensin-2; ACE, angiotensin converting enzyme (1.97); ANP, atrial natriuretic peptide (6.3); BNP, brain natriuretic peptide (3.28); PAM, peptidylglycine alpha-amidating monooxygenase (1.98); RXR, retinoid X receptor (2.28); PPAR, peroxisome proliferator-activated receptor-alpha (6.98); CTGF, connective tissue growth factor (2.67). The fold-change by real-time PCR is given in parenthesis and supports the relative changes observed by microarray analysis (Table 5).

expression in this case may be a compensatory response to the decreased contractility resulting from decreased cytosolic calcium. To answer this question, we have recently examined the contractility of the $\alpha 1^{+/-}$ hearts in response to external calcium concentration using a Langendorff preparation [14]. Lowering external calcium from 2mM to 1.5mM reduces cardiac contractility in hearts from the $\alpha 1^{+/-}$ mice, suggesting these hearts may be compromised for calcium [14]. In support of this assertion, we have recently re-examined the abundance of calcium in intracellular stores using a more sensitive calcium indicator than that previously used by James et al [3]. We observed less calcium in cardiac myocytes from $\alpha 1^{+/-}$ hearts compared to wild-type hearts (Yamamoto, Lingrel and Barry, unpublished). Changes in calcium handling genes may be a secondary response to the compensatory upregulation of the $\alpha 2$ isoform rather than a genetic reduction of the $\alpha 1$ isoform. The $\alpha 2$ isoform is functionally coupled to the Na/Ca exchanger and increasing expression of the $\alpha 2$ isoform would be expected to increase forward exchange mode of the Na/

Ca exchanger resulting in enhanced calcium extrusion from the cell. It seems reasonable that the L-type calcium channel (and the other ion transport genes) may be upregulated in the $\alpha 1^{+/-}$ hearts in a compensatory manner to bring in more calcium to cells that may already be compromised for calcium.

Activated renin-angiotensin system and natriuretic hormone transcripts in $\alpha 1^{+/-}$ mice

While compensatory changes in ion transporting genes may be expected, we also observed complex changes in genes regulating cardiovascular performance; changes which may be a direct result of $\alpha 1$ deficiency. For example, we observed an elevation of angiotensin converting enzyme (ACE; Table 3; log ratio of 2.24 in the $\alpha 1^{+/-}$ hearts compared to wild-type) and validated it by real-time PCR (1.97-fold increase; Table 5). Because ACE elevation suggested that the renin-angiotensin system is activated in the $\alpha 1^{+/-}$ mouse, we measured serum aldosterone and found it to be 2-fold elevated in the $\alpha 1^{+/-}$ mice (898 ± 77 pg/ml) compared to wild-type (480 ± 49

Fig. 5. Alterations in metabolism in the Na,K-ATPase $\alpha 1^{+/-}$ hearts. Numbers represent the following genes: 1, pyruvate dehydrogenase kinase-4 (3.75); 2, ATP citrate lyase (0.34); 3, succinyl CoA:3-oxo acid CoA transferase (2.28); 4, represents two genes: stearyl CoA desaturase-2 (0.24), long chain fatty acyl elongase (0.34); 5, isopentenyl-diphosphate delta isomerase (0.35). Also shown are ATP synthase (0.30) and uncoupling protein-3 (2.76). The average log ratio value from microarray analysis are given in parentheses, >1 is upregulation and <1 is down-regulation of gene transcription. $\uparrow$, upregulation of gene transcription and $\downarrow$, down-regulation of gene transcription. *Confirmed transcriptional change by real-time PCR.

Fig. 6. Upregulation of genes in $\alpha 1^{+/-}$ hearts involved in creatine-phosphate production. Numbers represent the following genes: 1, Na,Cl-dependent creatine transporter (2.18); 2, adenylate kinase isoforms-1 and -4 (2.56 and 2.23, respectively); 3, creatine kinase (1.87). The average log ratio value from microarray analysis is given in parentheses. $\uparrow$, upregulation of gene transcription. *Confirmed transcriptional change by real-time PCR.

pg/ml) (Figure 3, $p<0.05$). Our analyses indicate an activated renin-angiotensin system in the $\alpha 1^{+/-}$ mice, which is further supported by the observation that kidneys from the $\alpha 1^{+/-}$ mice have elevated renin mRNA levels (James and Lingrel, unpublished). Activation of the renin-angiotensin system is generally considered a response to

volume regulation or decreased blood pressure, and we have found that the $\alpha 1^{+/-}$ mice do indeed display low blood pressure and low cardiac contractility. Under these conditions, then, there appears to be an appropriate compensatory transcriptional activation of the renin-angiotensin-aldosterone axis that is working to restore or maintain fluid and pressure homeostasis (Figure 4, left side) which is most likely a genomic response stemming directly from depressed expression of the $\alpha 1$ isoform.

We subsequently examined the microarray list for genes involved in volume and blood pressure regulation. Importantly, our analyses indicated an activated natriuretic hormone response in $\alpha 1^{+/-}$ mice (Figure 4, right side). One of the more upregulated genes is brain natriuretic peptide (BNP; 3.12 log ratio by microarray; 3.28-fold increase by real-time PCR; Tables 2 and 5). We also detected a 13.5 fold increase atrial natriuretic peptide (ANP) levels by real-time PCR (as opposed to the microarray analysis showing only a 1.3 log ratio; Table 5). Interestingly, the enzyme that processes ANP

to its active form, peptidylglycine alpha-amidating monooxygenase (PAM) is upregulated 3.38 log ratio by microarray and 1.98-fold change by real-time PCR (Tables 2 and 5). ANP and BNP, made in the atria and ventricle, respectively, are usually secreted to reduce extracellular fluid and blood pressure through a variety of cardiovascular and renal mechanisms. For example, BNP acts to antagonize the action of aldosterone and reduce intravascular volume and pressure to help control cardiac output [15, 16]. Both ANP and BNP also act as vasodilators to decrease peripheral vascular resistance. Since the observed increase in natriuretic hormones levels would be considered inappropriate in the face of the decreased blood pressure in the $\alpha 1^{+/-}$ mice, we can speculate that they represent defective secondary signaling during $\alpha 1$ isoform deficiency, and that the stimulated renin-angiotensin system represents a response to try to restore pressure and volume. It is furthermore interesting to speculate that the failure of the renin-angiotensin system to fully compensate may be related to the down-regulation of adenosine deaminase seen in the $\alpha 1^{+/-}$ mice (0.17 log ratio value). Adenosine is an important signaling molecule in the kidney which acts to suppress renin secretion [17, 18]. Decreases in adenosine deaminase activity would be expected to reduce the extracellular breakdown of adenosine, thereby increasing local levels of inhibitory adenosine. Thus, although the renin-angiotensin system is stimulated in the $\alpha 1^{+/-}$ mice, it is possible that adenosine deaminase deficiency prevents it from being sufficiently elevated to fully restore volume and pressure homeostasis. The down-regulation of this enzyme may be part of a defective secondary signaling in the $\alpha 1^{+/-}$ mice similar to that suggested for the elevated natriuretic hormones ANP and BNP.

Interestingly, the neurohormonal and renin-angiotensin systems are activated at several biological levels: serum hormones (aldosterone), tissue hormones (ANP, BNP), processing enzymes (PAM) and transcription factors (RXR, PPAR). It is important to point out that many of these changes observed by microarray analysis were confirmed by real-time PCR (Table 5).

Altered transcriptional responses of metabolic genes in $\alpha 1^{+/-}$ hearts

Because cardiac output is dependent upon the metabolic energy status of the cardiomyocyte, we examined the microarray data for transcriptional changes in metabolic genes. Collectively, the transcriptional responses of several genes in the $\alpha 1^{+/-}$ hearts suggest

that fatty acid oxidation is enhanced compared to wild-type (Figure 5). Pyruvate dehydrogenase kinase-4 (PDHK4) is upregulated (log ratio of 3.75; 1, Figure 5; Table 3), which functions to phosphorylate pyruvate dehydrogenase. Phosphorylation of pyruvate dehydrogenase inactivates it, thereby blocking metabolic flow from glycolysis to acetyl CoA. PDHK4 is the key enzyme in determining fuel selection for the cell [19]. The inhibition of pyruvate dehydrogenase by PDHK4 would lead to the majority of acetyl CoA leading into the Krebs cycle for ATP production coming from β -oxidation rather than pyruvate (formed from glycolysis). In fact, many of the observed changes in transcription suggest an increase in fatty acid oxidation (4 and 5, Figure 5) and ketone acid utilization (3, Figure 5) in the $\alpha 1^{+/-}$ hearts (Table 3). At the same time we observed down-regulation of genes required for fatty acid synthesis in the $\alpha 1^{+/-}$ hearts (Tables 3 and 5; Figure 5). ATP citrate lyase (2, Figure 5), long chain fatty acyl elongase and stearoyl-CoA desaturase-2 (4, Figure 5) all function in fatty acid biosynthesis and are regulated by the same transcription factors (sterol regulatory element binding proteins, SREBP's) [20]. Each of these genes was found to be down-regulated in the $\alpha^{+/-}$ hearts: 0.3, 0.34 and 0.24 log ratios, respectively by microarray (Table 3). No statistically significant change in SREBP expression was found by the microarray analysis. ATP citrate lyase was also shown to be down-regulated 0.81-fold using real-time PCR (Table 5). Overall, our data indicate that $\alpha 1$ isoform deficiency results in upregulation of a transcriptional program to increase fatty acid oxidation.

In fact, we observe the upregulation of transcriptional pathways involved in metabolism. The retinoid X receptor-gamma (RXR) is a well-known transcription factor that transactivates genes involved in fat metabolism and works as a heterodimer with peroxisome proliferators-activated receptor (PPAR). Interestingly, the $\alpha 1^{+/-}$ hearts showed a significant upregulation of both RXR- γ and PPAR- α (Table 5, Figure 4). Known target genes of RXR and PPAR include BNP and PDHK4, whose upregulation in the $\alpha 1^{+/-}$ hearts supports the notion that RXR/PPAR may be actively trans-activating genes. As mentioned, PDHK4 is upregulated during times of enhanced fatty acid oxidation. Another gene regulated by PPAR in adult rat heart is uncoupling protein-3 (UCP-3). UCP-3 is also upregulated under conditions of elevated free fatty acids [21] and is elevated in the $\alpha 1^{+/-}$ hearts. An increase in UCP-3 expression is associated with a shift in substrate utilization (under glucose-limited

conditions) to favor lipids as the predominant metabolic fuel [22]. The upregulation of these transcriptional pathways further indicates a demand for increased energy metabolism, probably in an attempt to maintain cardiac output.

Further evidence of increased metabolic demand is the upregulation of genes involved in creatine-phosphate production (Figure 6): the creatine transporter (log ratio 2.18; 1, Figure 6), adenylate kinases 1 and 4 (log ratios 2.56 and 2.23, respectively; 2, Figure 6) and creatine kinase (log ratio 1.87 by microarray and 2.62 fold-change by real-time PCR; 3, Figure 6). High energy phosphate molecules such as creatine-phosphate are critical sources for supplying cardiomyocytes with fuel for cellular processes. Creatine is transported into the cardiac myocyte via the sodium- and chloride-dependent creatine transporter and it is upregulated in the $\alpha 1^{+/-}$ hearts. Next, the ATP used as a substrate to phosphorylate creatine is generated from the enzymatic reaction catalyzed by adenylate kinase and two adenylate kinase isoforms (1 and 4). It is significant that these genes are upregulated in the $\alpha 1^{+/-}$ hearts because creatine is phosphorylated by creatine kinase, the muscle-specific form of which is also upregulated in the $\alpha 1^{+/-}$ hearts. Taken together, upregulation of creatine transport, adenylate kinase and creatine kinase would be expected to enhance creatine-phosphate production in the cell. Recent studies of congestive heart failure report an upregulation of muscle-specific creatine kinase, suggesting that this response is a survival mechanism by the heart to obtain high energy source [23, 24].

The increase in energy metabolism genes was an unexpected genomic response in genetically reduced $\alpha 1$ mice because the Na,K-ATPase utilizes ~25% of the cells' ATP to transport Na and K ions against their gradients. One might expect excess energy available with reduced Na,K-ATPase expression, resulting in decreased, not increased, energy metabolism. Since this was not the case in the $\alpha 1^{+/-}$ mice, these changes may be a secondary compensation mechanism possibly in response to decreased cardiac contractility.

Antioxidant enzymes elevated in the $\alpha 1^{+/-}$ hearts

Finally, we also noted that glucose-6-phosphate dehydrogenase (G6PDH) transcripts are down-regulated in the Na,K-ATPase $\alpha 1^{+/-}$ mice. This enzyme has recently been shown to mediate the redox status of adult cardiac myocytes and protect the heart from reactive oxygen species [25, 26]. Two important antioxidant enzymes,

glutathione peroxidase-3 and glutathione transferase are also upregulated in the $\alpha 1^{+/-}$ hearts. Furthermore, the $\alpha 1^{+/-}$ hearts display increased expression of vascular endothelial growth factor-A (VEGF-A) and the VEGF receptor, which protect cells from ischemic death [27]. These results indicate that hearts from these mice could be undergoing an increase in oxidative stress, which has been implicated in the progression to heart failure [28]. This could also be a secondary response to oxidants generated by the increase in fatty acid metabolism. In a mouse model, increased anti-oxidant capacity is also associated with heart hypertrophy caused by energy metabolic disorder [29]. Although we have found no evidence of hypertrophy in these mice, oxidative stress may play a role in their reduced cardiac performance.

In summary, we have demonstrated that down-regulation of the Na,K-ATPase $\alpha 1$ isoform by gene-targeting resulted in diverse alterations of gene expression. While the depressed cardiac contractility observed in the $\alpha 1^{+/-}$ mice is a relatively mild phenotype, we observed a surprisingly high number of transcriptional changes occurring in hearts from these mice. For example, $\alpha 1^{+/-}$ hearts instigated transcriptional changes to restore cytosolic calcium levels in an effort to maintain cardiac contractility. It also appears that the renin-angiotensin system and the natriuretic hormone response are activated as a direct result of depressed $\alpha 1$ expression. While both of these pathways help to regulate blood pressure in opposite directions, the physiological consequence of these genetic changes is lower blood pressure in the $\alpha 1^{+/-}$ mice compared to wild-type. Furthermore, increased energy metabolism was suggested in the $\alpha 1^{+/-}$ hearts, as genes in the fatty acid oxidation pathway were upregulated, as well as those genes in creatine-phosphate synthesis. We also noted increased antioxidant capacity in the $\alpha 1^{+/-}$ hearts, which could indicate a predilection towards advanced cardiac pathologies.

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