Published in final edited form as:

J Mol Cell Cardiol. 2008 January ; 44(1): 69–75.

Selective Translation of mRNAs in the Left Ventricular Myocardium of the Mouse in Response to Acute Pressure Overload

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Abstract

During pressure overload hypertrophy, selective changes in cardiac gene expression occur that regulate growth and modify the structural and functional properties of the myocardium. To determine the role of translational mechanisms, a murine model of transverse aortic constriction was used to screen a set of specified mRNAs for changes in translational activity by measuring incorporation into polysomes in response to acute pressure overload. Candidate mRNAs were selected on the basis of two main criteria: 1) the 5'-untranslated region of the mRNA contains an excessive amount of secondary structure ($\Delta G < -50$ kCal/mol), which is postulated to regulate efficiency of translation, and 2) the protein product has been implicated in the regulation of cardiac hypertrophy. After 24 hours of transverse aortic constriction, homogenates derived from the left ventricle were layered onto 15%-50% linear sucrose gradients and resolved into monosome fractions (messenger ribonucleoprotein particles) and polysome fractions by density gradient ultracentrifugation. The levels of mRNA in each fraction were quantified by real-time RT-PCR. The screen revealed that pressure overload increased translational activity of 6 candidate mRNAs as determined by a significant increase in the percentage of total mRNA incorporated into the polysome fractions. The mRNAs code for several functional classes of proteins linked to cardiac hypertrophy: the transcription factors *c-myc*, *c-jun* and MEF2D, growth factors VEGF and FGF-2, and the E3 ubiquitin ligase MDM2. These studies demonstrate that acute pressure overload alters cardiac gene expression by mechanisms that selectively regulate translational activity of specific mRNAs.

Keywords

Cardiac Hypertrophy; Gene Expression; Protein Biosynthesis; Translation; Polyribosomes; messenger RNA; 5' Untranslated Regions

INTRODUCTION

Pressure overload of the left ventricle (LV), which is generated by diseases such as systemic hypertension and aortic stenosis, causes a sustained increase in systolic wall stress that triggers

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hypertrophic growth [1,2]. Hypertrophy of the myocardium is produced by a global increase in protein synthesis that facilitates accumulation of myofibrils, mitochondria and other primary components of the cardiac muscle cell (cardiocyte) [3,4]. Translational mechanisms play a major role in accelerating the rate of protein synthesis in response to pressure overload by increasing the activity (efficiency) and amount (capacity) of the translation machinery, i.e., ribosomes and associated components such as eukaryotic initiation factors (eIFs) [5,6]. Given that availability of mRNA is not a rate-limiting determinant for protein synthesis, these mechanisms are generally non-selective with respect to translation of individual mRNAs. Consequently, synthesis of constitutive proteins such as myosin increase in proportion to total protein synthesis because the corresponding mRNAs compete effectively for ribosomes and are translated at high efficiency, both at steady-state and during periods of hypertrophic growth [7].

In addition to increased protein content, the hypertrophic phenotype is generated by selective changes in gene expression that regulate growth and modify the structural and functional properties of the cardiocyte [4]. Transcriptional mechanisms are postulated to direct these changes, thereby producing the hypertrophic gene program that is essential for the development of compensatory growth [8]. However, changes in specific mRNA levels during cardiac hypertrophy appear to be limited to a relatively small number of genes. For example, a microarray containing 12,488 known genes and expressed sequence tags identified 269 genes that were up-regulated significantly after 10 days pressure overload hypertrophy, and 160 genes that were down-regulated [9]. Most of these mRNAs were induced or repressed in the range of 1.5- to 2-fold, which suggests that expression of many genes is also dependent on how efficiently each mRNA is translated relative to the total mRNA pool.

The 5'-untranslated region (5'-UTR) of mRNA has structural features that function as one of the main determinants of translational efficiency [10,11]. A large majority of mRNAs have a 5'-UTR that is relatively short (approximately 100 nt in length) and/or contains a minimal amount of predicted secondary structure ($\Delta G > -50$ kCal/mol). These mRNAs are classified as "strong" because the 5'-UTR is posited to facilitate efficient translation during the initiation step. A smaller subset of mRNAs (approximately 10%) has a more complex 5'-UTR that typically contains one or more of the following features: length > 200 nt, G+C content $> 65\%$ and/or excessive secondary structure ($\Delta G < -50$ kCal/mol). These mRNAs are classified as "weak" because greater structural complexity of the 5'-UTR renders it less efficient with respect to translation. The 5'-UTR of weak mRNAs is hypothesized to function as an additional mechanism to limit synthesis of the corresponding proteins to periods of growth, a time when activity and/or amount of the translation machinery is increased [12].

In this study, an acute, murine model of transverse aortic constriction (TAC) was used to screen for specific mRNAs that are regulated by translational mechanisms in response to LV pressure overload. Specifically, we selected candidate mRNAs from a 5'-UTR database on the basis of two main criteria: 1) the 5'-UTR contains features that are predictive of weak mRNAs with respect to translational efficiency, and 2) the protein product has been implicated in the regulation of cardiac hypertrophy. For each candidate mRNA, the percentage of the total pool active in translation was determined by measuring incorporation into polysome fractions. These studies have identified a diverse set of candidate mRNAs with increased translational activity in response to acute LV pressure overload, all of which code for proteins involved in cardiac hypertrophy.

MATERIALS AND METHODS

Transverse Aortic Constriction (TAC) Model of LV Pressure Overload

TAC was created using the FVB strain of mice as described before [13]. A baseline body mass was obtained, and the mice were anesthetized with 1.5-2% isofluorane in oxygen. Mice were intubated and ventilated with a volume-cycled rodent respirator (Harvard Apparatus). A thoracotomy was performed and the transverse aorta was constricted by tying a 6-0 prolene suture around the vessel over a blunted 28-gauge needle. The needle was removed, which restored the lumen to the size of the 28-gauge needle, and created severe stenosis of the aorta as indicated by a pressure gradient of 92 ± 1 mm Hg (mean $\pm$ SE) across the suture. Sham-operated mice were subjected to a thoracotomy and a prolene suture was rubbed over the transverse aorta without constricting the lumen. After 24 h, mice were subjected to echocardiography using a 9.0 MHz transducer to verify an increase in the pressure gradient between the LV and the thoracic aorta. Echocardiography was used to measure LV thickness, ventricular dimension and mass. The mice were sacrificed after the pressure gradient was verified. LV mass and body weight were measured at the time of dissection [14]. The procedures involving animals were approved by the Institutional Animal Care and Use Committee, and all animals were treated and cared for in accordance with institutional guidelines.

Isolation of Polysome Fractions from Murine Myocardium

The RV of individual mouse hearts was dissected from the LV in phosphate buffered saline containing 100 μ g per mL cycloheximide to inhibit peptide elongation. A small portion of the LV was snap frozen for later extraction of total RNA using the Trizol® method (Invitrogen Life Technologies). The remaining LV was placed into ice-cold resuspension buffer consisting of 10 mM Tris-HCl (pH 7.5), 250 mM KCl, 10 mM MgCl₂, 0.5% Triton X-100, 2 mM dithiothreitol, 100 μ g/mL cycloheximide, 2 Units of RNasin (Promega). The LV was homogenized using a tissuemizer (Janke & Kunkel) by applying 4 pulses of 20 sec, with a 30 sec rest on ice between pulses. Tween/deoxycholate solution was added following homogenization to further solubilize membranes. After a 15 min incubation on ice, the homogenate was centrifuged at 12,000 $\times$ g to produce a post-mitochondrial supernatant. The supernatant was layered onto a 15-50% linear sucrose gradient in a buffer containing 200 mM Tris-HCl (pH 7.5), 2.5 M KCl, and 100 mM MgCl₂, and centrifuged at 100,000 $\times$ g for 100 min at 4° C. A density gradient fractionator (Teledyne Isco, Inc.) was used to isolate monosome fractions (messenger ribonucleoprotein particles and free ribosomes) and polysome fractions [15]. The RNA in each fraction was extracted using Trizol® reagent.

Measurement of Candidate mRNA Levels by Real-Time RT-PCR

RNA extracted from the gradient fractions was resuspended in 1 mM MgCl₂, treated with RQ1 DNase (Promega) and incubated at 37° C for 30 min. The DNase was subsequently heat inactivated at 90° C for 5 min. The relative concentrations of each candidate mRNA was quantified by real-time RT-PCR using a single step, SYBR® green assay system (Qiagen). A set of sequence-specific primers was designed and tested for each mRNA. Upon completion of each run, a temperature gradient, step-down program was run to monitor for primer-dimer formation. Amplicons produced by each primer set were verified as single bands of the correct size by electrophoresis of RT-PCR products on a 2% agarose gels.

Data Analysis

For each set of real-time RT-PCR reactions, a serial dilution of an RNA sample was used to generate a standard curve for extrapolation of relative mRNA concentrations. To correct for RNA recovery in each sample, the concentrations of each candidate mRNA were normalized

to the concentration of GAPDH mRNA. The percentage of polysome bound mRNA in each gradient was calculated and compared to the sham-operated controls. Total RNA derived from LV of TAC mice was plotted as a fold change over sham-operated controls. Differences in the mean values for sample concentrations were tested for statistical significance using a Student's T-test.

RESULTS

Selection Criteria of Candidate mRNAs

In this study, a TAC model of LV pressure overload was used to identify specific mRNAs that are regulated by translational mechanisms during cardiac hypertrophy. Candidate mRNAs were selected from a human, full-length mRNA database in which individual mRNAs have been separated into one of three classes using a classification and regression tree (CART) model [10]. Class I mRNAs are predicted to be relatively weak with respect to translational efficiency because the 5'-UTR contains one or more of the following structural characteristics: 1) a length greater than 200 nucleotides, 2) relatively high G+C content of 65% or greater, 3) excessive secondary structure as predicted by a ΔG of -50 kCal/mol or less. Table 1 is a list of 42 Class I mRNAs selected as candidates for investigation. The structural features of the 5'-UTRs are given in Table 2. For each candidate mRNA, the predicted free energy of formation (ΔG) was determined by mFOLD analysis using the full-length sequence of the 5'-UTR [16]. Some of the candidate mRNAs were selected on the basis of cardiac-specific expression or because they have been implicated in regulating cardiac hypertrophy. However, several Class I candidates were included in the study because they are involved in regulating growth of other cell types.

Establishment of Acute Time Point of LV Pressure Overload

The initial experiments were designed to determine the earliest time point at which the effects of pressure overload on translational activity of candidate mRNAs could be examined in the absence of secondary effects caused by anesthesia and surgery. Figure 1 shows time-dependent changes in the expression of three genes that normally are induced in response to pressure overload, namely atrial natriuretic factor (ANF), sodium calcium exchanger (NCX) and *c-jun*. The expression of ANF mRNA increased in a time-dependent manner in the TAC mice, but remained relatively constant in the sham-operated controls, even at the 24 h time point (Figure 1A). These results demonstrate that the TAC model was sufficient to produce a hypertrophic response and that stresses associated with anesthesia and surgery were minimal by 24 h. NCX mRNA levels remained constant over the first 48 h, although a modest increase in TAC mice was observed by 72 h. The levels of *c-jun* mRNA remained relatively stable in sham-operated and TAC mice between days 1 and 7. This result is consistent with previous studies showing that induction of *c-jun* mRNA in response to stress is rapid and transient in the heart and that expression of *c-jun* mRNA peaks within h after imposition of pressure overload. In the context of these studies, the constancy of *c-jun* mRNA expression confirmed the absence of non-specific stresses in the TAC model.

Cardiac Mass in Sham-operated versus TAC Mice

Equal numbers of mice were subjected to either TAC or sham operation. The data in Table 3 show that there were no significant differences in body weight, LV mass or LV mass/body weight ratios between sham-operated and TAC groups as measured 24 h after surgery.

Translational Activity of Candidate mRNAs

At 24 h after surgery, polysomes were isolated from the LV in order to measure the percentage of each candidate mRNA active in translation. The amount of polysome-bound mRNA was calculated as a percentage of its total pool of mRNA. For the initial screening of the candidates

listed in Table 1, RNA extracted from individual hearts was pooled into TAC and sham-operated groups in order to identify those candidate mRNAs with the greatest increase in translational activity. This initial screen identified 12 mRNAs that were mobilized into polysomes as exhibited by a TAC-induced increase in the percentage of polysome-bound mRNA (Figure 2). Next, changes in the distribution of the 12 candidate mRNAs were measured using RNA derived from individual hearts (Figure 3). These data show that translational activity of 6 candidate mRNAs were increased after 24 h of pressure overload as measured by an increase in the percentage of polysome-bound mRNA. These are the transcription factors, *c-jun*, *c-myc* and MEF2D, the growth factors FGF-2 and VEGF, and the p53 binding protein MDM2.

Effects of Pressure Overload on Total mRNA Levels

The amount of polysome-bound mRNA can be altered by changes in abundance of the total mRNA pool produced by transcriptional and/or post-transcriptional mechanisms. Therefore, we measured changes in the levels of the candidate mRNAs in the total RNA fraction after 24 h of TAC. Figure 4 shows relative mRNA levels in TAC mice normalized to sham-operated controls. Although small fluctuations in mRNA levels occurred after 24 h of pressure overload, only *c-myc* mRNA levels were significantly increased. For the other Class I candidate mRNAs, an increase in the percentage of polysome-bound mRNA in response to pressure overload is indicative of a selective increase in translational activity.

DISCUSSION

In adult cardiac muscle, approximately 85% of the ribosome pool is associated with polysomes, both at steady-state and during periods of hypertrophic growth [6]. This high degree of ribosome occupancy indicates that overall efficiency of protein synthesis is sustained normally at high levels, and that mRNAs compete for ribosomes in order to initiate protein synthesis [17]. Thus, during hypertrophic growth, the rate of total protein synthesis is accelerated by expanding the pool of ribosomes and associated components that are available for the initiation step of translation [5]. On the basis of these observations, one can conclude that the large majority of mRNAs are strong with respect to translation, and therefore constitute the general increase in cardiac protein synthesis. Nevertheless, it is likely that additional mechanisms are utilized in order to selectively increase the synthesis of proteins derived from mRNAs that are relatively weak with respect to translation. These studies demonstrate that the hypertrophic stimulus of pressure overload mobilizes specific mRNAs for translation by mechanisms that either improve the overall efficiency of translation or increase the stability of mRNA incorporated into polysomes. By screening candidate mRNAs for selective translation in response to acute pressure overload, the results underscore the value of looking beyond changes in mRNA abundance to determine mechanisms that control gene expression during different phases of hypertrophic growth.

In addition to an excessive amount of predicted secondary structure in the 5'-UTR, each of the mRNAs that exhibited increased incorporation into polysomes code for proteins implicated in cardiac hypertrophy. Several code for transcription factors. For example, *c-jun*, which functions as a component of the AP-1 transcription factor complex, is an immediate-early gene induced in response to pressure overload [18]. Its role in regulating the expression of hypertrophic genes such as skeletal α -actin has been well characterized [19]. MEF2D is a member of myocyte enhancer factor-2 family of transcription factors that are activated by Ca^{2+} /Calmodulin-dependent signaling pathways, and regulate expression of structural genes in the cardiocyte [20]. Although changes in MEF2 levels are relatively modest during cardiac hypertrophy, recent findings have linked MEF2 to expression of target genes associated with myocardial remodeling, for example cytoskeletal components and ion channels [21,22]. FGF-2

and VEGF are members of the heparin binding protein family of growth factors. FGF-2 is synthesized and secreted by muscle and non-muscle cells of the myocardium in response to myocardial stress, and it has a wide array of functions pertaining to inflammation, cardio-protection following ischemia-reperfusion injury, angiogenesis, cardiocyte growth and remodeling of the extracellular matrix [23]. Similarly, VEGF is an essential paracrine mediator of vascular remodeling in the pressure overloaded myocardium [24]. MDM2 is an E3 ubiquitin ligase that regulates degradation of proteins involved in growth and apoptosis. Overexpression of MDM2 has been shown to enhance cardiocyte survival under hypoxic conditions, while decreased expression impaired functional recovery from ischemia-reperfusion in isolated, perfused hearts [25]. Furthermore, it has been found to interact with TCAP, a sarcomeric protein important in the development of muscle hypertrophy [26]. These findings show that acute pressure overload mobilizes several functional classes of mRNA for translation, all of which have established roles during the process of cardiac hypertrophy, remodeling and survival.

These studies did not examine specific mechanisms by which incorporation of these mRNAs into polysomes was increased, but the most likely possibilities are either improved efficiency of translational initiation or translation-linked stability. To overcome constraints on cap-dependent initiation, RNA helicase activity is recruited to the cap to melt secondary structure in the 5'-UTR of the mRNA. This process is controlled by eIF4F, a complex composed of three core proteins: 1) eIF4E, which binds to the 7-methylguanosine cap on the 5'-end of mRNA, 2) eIF4A, which functions as an ATP-dependent RNA helicase, and 3) eIF4G, which contains binding sites for eIF4E and eIF4A, and functions as a molecular platform for assembling the eIF4F complex [27]. Affinity of eIF4F for the 7-methylguanosine cap can be modified by phosphorylation of eIF4E on Ser-209, which reduces its cap binding affinity as demonstrated *in vitro* [28]. Although the precise effects of eIF4E phosphorylation on translational efficiency of specific mRNAs have not been elucidated in cardiac muscle, we have shown in previous studies that eIF4E phosphorylation is increased in response to pressure overload hypertrophy [7,29]. Further evidence indicates that the mRNAs coding for MDM2, *c-myc*, *c-jun*, and FGF-2 contain Internal Ribosome Entry Sites (IRES), which are elements in the 5'-UTR that direct translational initiation independent of the 7mGppp cap [30-33]. The presence of an IRES could target specific mRNAs for translation in response to a growth stimulus by facilitating cap-independent initiation. However, the physiological relevance of IRES elements as a mechanism of translational initiation is controversial [34].

An alternative mechanism to account for the increased percentage of mRNA in polysomes is stabilization coupled to translational activity [35]. Once translation is initiated, protein components of the translation machinery cause mRNA to assume a circular conformation by positioning the cap region of the 5'-end in close proximity to the PolyA tail on the 3'-end. Consequently, incorporation of mRNA into polysomes can promote stabilization by limiting accessibility of enzymes that trigger mRNA degradation, specifically decapping enzymes and deadenylases [36]. Polysomes also regulate stability of mRNA by other mechanisms that involve activity of constituent proteins. For example, recent findings in adult cardiocytes have shown that the eIF4E kinase Mnk1 functions by stabilizing the pool of *c-jun* mRNA incorporated into polysomes [37].

The candidate mRNAs were screened for polysome incorporation after 24 h of TAC, a time point that proved feasible for examining the early effects of pressure overload on cardiac gene expression. The rationale for choosing an acute time point is that increased translational activity would likely occur as an initial response to pressure overload. This would enable existing myocardial mRNAs to be utilized more efficiently prior to any adaptive changes in mRNA abundance. However, further studies will be required to address the following limitations of this approach for screening candidate mRNAs: 1) Changes in translational activity of some

candidate mRNAs could have occurred at earlier or later time points, for example, during sustained pressure overload hypertrophy; 2) Changes in expression of the corresponding proteins derived from the mRNAs identified by this screen have not been determined. Nevertheless, these studies demonstrate that the stimulus of pressure overload increased translational activity of mRNAs that code for several functional classes of proteins linked to hypertrophy. The underlying mechanisms that selectively regulate translation of these mRNAs may be essential to initiate cardiac growth and remodeling.

Acknowledgements

We wish to thank Elizabeth Hapke and Daisy Dominick for their excellent technical assistance. This work was supported by National Institutes of Health Grant PO1 HL-48788 and the Research Service of the Department of Veterans Affairs.

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Figure 1.

Time course of expression for marker genes of cardiac hypertrophy. Levels of ANF mRNA, Na⁺-Ca²⁺ Exchanger (NCX) mRNA and *c-jun* mRNA were quantified in total RNA samples derived from LV at the indicated time points after surgery. Values for TAC and Sham-operated mice are expressed as the fold increase relative to non-operated controls.

Figure 2.

Initial screen for translationally regulated mRNAs. Equal volumes of mRNA were pooled to complete an initial screen of the candidate mRNAs. Shown are the 12 mRNA candidates with the greatest increase in percentage of polysome-bound mRNA. Values are expressed as the percentage of each mRNA pool that was polysome-bound after 24 h in sham-operated and TAC mice.

Figure 3.

Secondary screen for translationally-regulated mRNAs. RNA samples derived from individual hearts were used to re-screen the 12 candidate mRNAs identified in Fig. 2. Values are expressed as the percentage of each mRNA pool that was polysome-bound after 24 h in sham-operated and TAC mice; mean $\pm$ SE, * $p < 0.02$ as determined by a Student's t-test.

Figure 4.

Changes in the levels of candidate mRNAs in response to pressure overload. Total mRNA levels were quantified by real-time RT-PCR. Values in TAC mice were normalized to the corresponding values in Sham-operated controls; mean $\pm$ SE, * $p < 0.05$ as determined by a Student's t-test.

Table 1

Candidate mRNAs

Adv.	Protein
Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase
ACE1	Angiotensin Converting Enzyme 1
AGTR1A	Angiotensin Receptor 1A
Bcl10	B-cell leukemia/lymphoma 10
Cal-A	Calcineurin A α subunit
CCR6	Chemokine Receptor 6
c-jun	c-jun protooncogene
COPA	Coatomer Protein A
c-myc	c-myc protooncogene
c-Src	c-Src
CKAP	Cytoskeletal Associated Protein
MDM2	Double Minute p53 Binding Protein
ENDRA	Endothelin Receptor Subtype A
FGF2	Fibroblast Growth Factor 2
GATA4	GATA Binding Protein 4 Transcription Factor
Grb14	Growth Factor Receptor Bound Protein 14
IGFBP1	Insulin Like Growth Factor Binding Protein I
IGFIR	Insulin Like Growth Factor 1 Receptor
Kir6.1	Inward Rectifying Potassium Channel 6.1
LCaChan	L-Type Calcium Channel
MAD4	Max-Associated Protein 4 Transcription Repressor
MEF2D	Myocyte Enhancing Factor 2D Transcription Factor
MAPKK3	Mitogen Activated Protein Kinase Kinase 3
NFAT	Nuclear Factor of Activated T Cells
NFkB	Nuclear Factor κ B
NRF	Nuclear Respiratory Factor
ODC	Ornithine Decarboxylase
PITPN	Phosphatidylinositol Transfer Protein
PPP1R2	Protein Phosphatase Inhibitory 1 Regulatory Subunit 2
PTPCAAX	Protein Tyrosine Phosphatase 4a1
PP2ARe	Protein Phosphatase 2A Regulatory Subunit
PTPRM	Protein Tyrosine Phosphatase Receptor M
PIM1	Proviral Integration Site 1
RGS5	Regulator of G-Protein Signalling
RAR γ	Retinoic Acid Receptor γ
STK25	Serine Threonine Kinase 25
SRF	Serum Response Factor
TIMP2	Tissue Inhibitor of Matrix metalloproteinase 2
TOP1	Topoisomerase I
UCP2	Uncoupling Protein 2
VEGFA	Vascular Endothelial Growth Factor A
CACNG	Voltage Dependent Calcium Channel γ 2

Table 2

Structural Features of Candidate mRNAs

	<u>5'UTR Length</u>	<u>% GC</u>	<u>AG</u>		<u>5'UTR Length</u>	<u>% GC</u>	<u>AG</u>
ACE1	1505	67	-688	MDM2	305	67	-135
AGTR1A	684	33	-132	MEF2D	391	70	-161
Alc10l	1494	56	-588	MKK3	337	60	-125
Bcl 10	706	61	-283	Mir	395	77	-166
CACNG	283	34	-48	NEAT	240	80	-86
Cal A	327	76	-186	NRF	225	61	-89
CCR6	551	46	-139	ODC	347	65	-151
c-Jun	974	65	-421	PTPN	231	84	-118
CKAP	273	66	-134	PIM1	364	75	-139
c-myc	558	65	-249	PTPCAAX	1153	54	-406
COPA	466	62	-171	PTPRM	425	43	-106
c-Src	350	65	-123	PP2ARe	591	70	-215
ENDRA	355	64	-130	PP1R2	226	68	-141
FGF2	466	80	-188	RARG2	613	60	-210
GATA4	301	51	-85	RGS5	278	51	-88
Grb14	540	79	-254	SRF	362	79	-195
IGF1R	1040	69	-519	STK25	246	77	-116
IGFBP4	285	82	-127	TIMP2	302	82	-137
Kir 6.1	300	65	-118	TOP1	246	72	-83
LCaChan	1411	34	-271	UCP2	380	57	-119
MAD4	116	87	-61	VEGFA	1038	66	-477

Table 3**Cardiac Mass in Sham-operated and TAC Mice**

Group	Age at Sacrifice (weeks)	Body Wt (g)	LV Wt (mg)	LV/Body Wt (mg/g)
Sham	11.1 ± 0.6	24.0 ± 0.7	83.6 ± 2.8	3.5 ± 0.1
TAC	10.4 ± 0.4	22.9 ± 0.7	80.0 ± 1.5	3.5 ± 0.1

Values are the mean ± SE, n = 12 mice per group.