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Hypertrophic Gene Expression Induced by Chronic Stretch of Excised Mouse Heart Muscle

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Abstract: Altered mechanical stress and strain in cardiac myocytes induce modifications in gene expression that affects cardiac remodeling and myocyte contractile function. To study the mechanisms of mechanotransduction in cardiomyocytes, probing alterations in mechanics and gene expression has been an effective strategy. However, previous studies are self-limited due to the general use of isolated neonatal rodent myocytes or intact animals. The main goal of this study was to develop a novel tissue culture chamber system for mouse myocardium that facilitates loading of cardiac tissue, while measuring tissue stress and deformation within a physiological environment. Intact mouse right ventricular papillary muscles were cultured in controlled conditions with superfusate at 95% O_2/ 5% CO_2, and 34°C, such that cell to extracellular matrix adhesions as well as cell to cell adhesions were undisturbed and both passive and active mechanical properties were maintained without significant changes. The system was able to measure the induction of hypertrophic markers (BNP, ANP) in tissue after 2 hrs and 5 hrs of stretch. ANP induction was highly correlated with the diastolic load of the muscle but not with developed systolic load. Load induced ANP expression was blunted in muscles from muscle-LIM protein knockout mice, in which defective mechanotransduction pathways have been predicted.

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1 Introduction

Cardiac hypertrophy is a physiological adaptation of the heart in response to an increase in biomechanical stress. It is also a major independent risk factor of death, underling various cardiovascular maladies including heart failure and sudden death [13]. Myocardial hypertrophy is characterized by an increase in cardiomyocyte size, increased protein synthesis, and re-activation of embryonic genes for contractile proteins and natriuretic peptides (atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP)) [13, 38]. Cardiomyocytes are dynamic systems that respond to mechanical load through the mechanisms of mechanotransduction, where forces acting on transmembrane and/or intracellular mechanosensors are transduced into a series of biochemical signals that induce hypertrophy [24, 54]. Defects in the mechanotransduction process may lead to the development of dilated cardiomyopathy or heart failure. Understanding some of these mechanisms, both mechanical and biochemical, may help identify potential therapeutic strategies for the prevention and treatment of heart failure or dilated cardiomyopathy.

Several in vivo and in vitro models of load-induced hypertrophy have been previously described. Pulmonary and aorta coarctation are common in vivo techniques utilized to characterize the development of left and right ventricular hypertrophy [2]. In vivo experimental models of load induced hypertrophy increase hemodynamic stress, but other variables, such as ischemia, neurohumoral factors, inflammation, and growth factors may also play a significant role in stimulating myocardial hypertrophy in these models. Increased mechanical load produced by elevated

perfusion pressures in a Lagendorff setup using isolated whole rat hearts, neonatal pig hearts, and ferret hearts has been used to stimulate the development of myocardial hypertrophy in an in vitro environment [3, 4, 23, 35]. Although such a system provides for a more controlled environment, it is limited to a few species and can only maintain tissue viability for a couple of hours. Furthermore, perfusion pressures in the coronaries are increased and this may have unknown effects on the myocardium. More commonly, load induced cardiac hypertrophy has been studied in vitro using neonatal rodent ventricular myocytes as a model system [25, 40, 48, 49]. In these systems the in vivo hypertrophic phenotype of cardiac myocytes is induced by applying a static uniaxial stretch of 10- 20% to neonatal cardiac cells grown on a stretchable substrate. Recently, the equibiaxial stretch system has been refined with the introduction of a more physiological, multiaxial stretcher [14]. Cyclical stretch of neonatal cardiac myocytes grown on flexible substrates is also becoming a more popular technique [10, 44]. While many of the characteristics of in vivo load induced hypertrophy are preserved in this model system (example: increase in protein synthesis, induction of immediate early genes, and re-expression of 'fetal genes'), neonatal cardiomyocytes are phenotypically different from adult cardiomyoctes, having a different morphology and retaining the capacity to divide [18]. There are several studies that have imposed mechanical loads onto isolated adult cardiomyocytes to study hypertrophy, but these studies are limited to a few animal species (rat and feline) [22, 30, 41]. This model may not reflect the stretch induced behavior of intact adult cardiac tissue, which is composed of cardiac myocytes and non-cardiac cells such as fibroblasts and endothelial cells. Additionally, it is difficult to measure systolic and diastolic mechanical properties of cultured myocytes [19]. For this reason, it is difficult to assess if mechanical properties may affect or be affected by the various stages of hypertrophy.

Finally, papillary muscles, which are extensions of the myocardial wall containing approximately 75% by volume cardiac muscle cells [31], have also been used as model systems to study load induced hypertrophy of intact adult cardiac tissue [6, 8, 17, 20, 36]. For these in vitro studies, a static load is applied to rabbit, ferrite, or rat papillary muscles and the rate of protein synthesis and/or mRNA synthesis is measured as a function of tissue tension at specified time points. These experiments allow for the control of systolic and diastolic tissue stress and strain, modeling the adult heart more closely to physiological conditions.

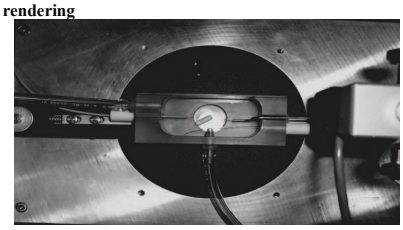
Genetically modified mouse models are powerful tools in science used to investigate the roles of specific proteins in load-dependent hypertrophic signaling of the heart, in modulating the mechanical properties of cardiac tissue, or both [24, 26, 53]. The main goal of this study was to develop a system in which the application of uniaxial stresses to mouse right ventricular papillary muscles can induce a hypertrophic response that is measurable at the molecular level, while simultaneously assessing the mechanical properties of the tissue. The system was developed for the purpose of utilizing genetically engineered mouse models of human cardiovascular disease to study the mechanisms of mechanotransduction in the heart.

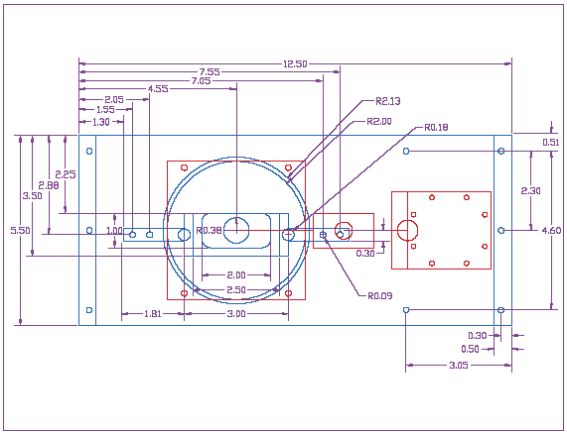
2 Methods

2.1 Experimental Protocol

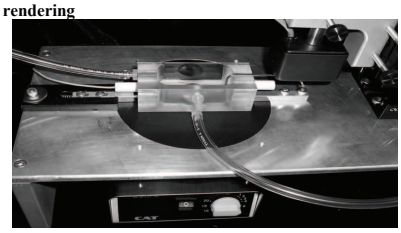
Experimental procedures were performed according to the AAALAC animal guidelines and the guidelines for the care of and use of laboratory animals of the University of California, San Diego. Male C57BL/6 mice (Charles River) or muscle specific LIM protein knockout mice with the C57BL/6 background [24] (ages: 2-8 weeks) were anesthetized with Isoflurane. After cervical dislocation, the chest was opened and the heart was arrested by intracardiac injection of low calcium (1mM), high potassium (15mM) cardioplegic solution containing 20mM of 2,3-butanedione monoximen (BDM). The heart was rapidly removed, cannulated, and retrogradely perfused through the aorta with 10mM Hepes-buffered (pH=7.36) solution containing (mM): 137.2 NaCl, 15.0 KCl, 1.2 MgCl2, 2.8 Na Ac-

A. Top View and AutoCAD rendering





B. Side View and AutoCAD rendering



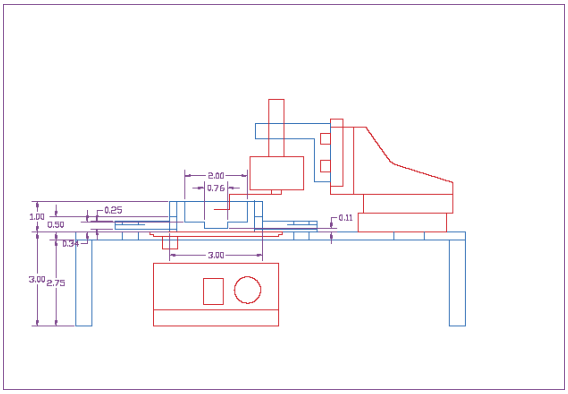


Figure 1: The RV papillary muscle culture chamber and mechanics apparatus. Muscle is secured between a basket and titanium hook. Muscle length is adjusted using a Newport CM-12CC actuator. Muscle forces are measured using an Isometric Harvard Apparatus force transducer (724490). Culture media is continuously circulated into and out of the muscle bath through inlet and outlet chamber ports. An ECM2 magnetic stirrer provides for a well stirred environment within the chamber for optimal diffusion of nutrients, oxygen, and wastes. The culture media is maintained at 34°C with a heated stage insert with temperature controller (World Precision Instrument: WPI-14471).

etate, 10 Taurine, 1.0 CaCl2, 10 Glucose, and 20 BDM in equilibrium with 100% O2. The right ventricle was opened and a right ventricular (RV) papillary muscle was dissected free.

RV papillary muscles were mounted horizontally in a custom made, biocompatible, polycarbonate culture chamber (**figure 1**) containing the Hepes-buffered solution used for dissection. Muscles were mounted between a stainless steel basket shaped extension attached to an Isometric Harvard Apparatus force transducer (724490) mounted onto an actuator-controlled (Newport CM-12CC), high precision, linear modular ball bearing stage (Newport 460P-xyz), and a stationary titanium hook. Oxygen was pumped over the top of the solution through an inlet tube in the chamber top during the mounting procedure, to prevent hypoxia induced ANP and BNP induction, which may mask out any stretch induced natriuretic peptide stimulation. All portions of the system were steam sterilized prior to each experiment. All solutions were filter sterilized. After the muscle was securely mounted, the Hepes-buffered solution was exchanged for a modified M199 cell culture media containing (mM): 2.0 L-carnitine, 5.0 creatine, 5.0 taurine, 2.0 L-glutamine, 0.2% albumin, 100 IU/ ml penicillin, 0.1 mg/ml streptomyocin, 10mM Hepes, and 20U/l of insulin in equilibrium with 5% CO2 and 95% O2 [19]. A 100ml reservoir of modified M199 media was maintained at 37°C and a pH of 7.4 and was pumped into and out of the culture chamber through in and outflow chamber ports. Using this method and a microincubator heated stage insert with temperature controller (World Precision Instrument: WPI-14471) the temperature of the muscle chamber was maintained at 34°C. For optimal diffusion of nutrients, oxygen and waste to and from the tissue a well stirred environment was sustained in the tissue bath using a magnetic stirrer (ECM2) and a 5mm Teflon stirring bar.

The muscle was stimulated to contract by way of the titanium hook and a platinum electrode positioned within close proximity of the muscle's base. Muscles were stretched to 10% of Lmax, where Lmax was defined as the length of the muscle at which the muscle produces the greatest developed systolic tension, and stimulated at 0.1 Hz for one hour. After the muscle had equilibrated and forces had stabilized, the stimulation frequency was increased to 0.5 Hz. Muscles treated with high mechanical loads were stretched to ∼90% of Lmax. Control muscles were stretched to ∼10% of Lmax. Muscle length changes were acquired with a linear voltage displacement transducer (Omega LD310-10). To measure local muscle deformations, the surface of the muscle was marked with titanium dioxide particles (**figure 3**). Local deformations of the muscle were recorded by a CCD camera (COHU Inc.). Muscle dimensions were acquired by video capture, calibrated with a phantom of known dimensions placed at the same focal length as the specimen. The cross sectional area of the muscle was assumed to be an ellipse.

Using this experimental setup, uniaxial muscle forces were recorded while pacing the muscle and during continuous stretching of the muscle up to Lmax. Recorded video frames of muscle deformation were synchronized with acquired force data. Diastolic data points were chosen before the time of twitch activation. Developed systolic tension in the muscle was calculated as the total force at the peak of a contractile twitch at a given local strain minus resting force at the same strain; this assumes that the elastic element of the muscle, which is assumed to be the determinant of diastolic tension, is in parallel with the contractile element, which is the determinant of the developed systolic tension.

Local lagrangian uniaxial strain measurements (E=1/2(λ^2-1), where λ is the stretch ratio) were calculated from the local tissue stretch ratios, using slack length of each muscle as its reference. The stretch ratio was determined from the displacement of two dots located along the long-axis fiber direction of the tissue. Cauchy stresses were calculated by dividing acquired force data by the initial cross sectional area of each muscle.

2.2 Quantitative-PCR Techniques

At the end of stretch, the muscles were immediately submerged in RNA later solution (Quia-

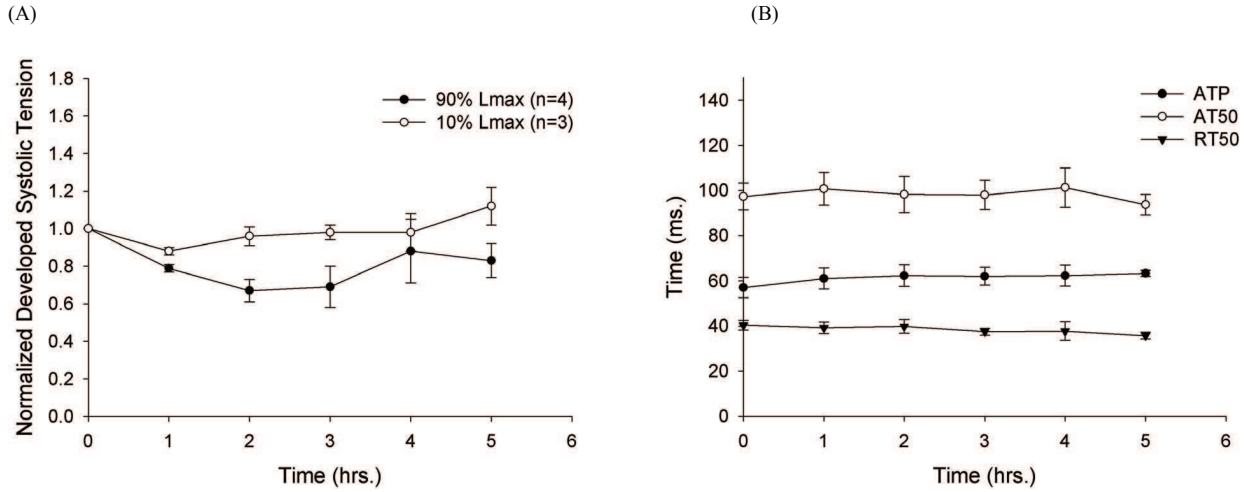


Figure 2: Stable systolic tissue mechanics indicate tissue viability was preserved for duration of experiment. (**A**) Average time course of systolic tension developed over first 5 hours of culture. Data was normalized by initial developed systolic tension. (**B**) Time course of time from activation to peak tension developed (ATP), time from activation to 50% relaxation (AT50), and 50% relaxation time (RT50) of right ventricular papillary muscles stretched to 90% of Lmax. (n=4) Data are mean +/- SEM.

gen), which functions to stabilize RNA in intact tissue samples and contains RNase inactivating reagents. The RV papillary muscles were completely isolated from their attachments, the valve and septal chunk. Muscles were homogenized with a Polytron and RNA was extracted as described (Quiagen's RNeasy Micro RNA purification kit). Given that total RNA extraction from these small specimens is low ($\sim$10ng/ul) and that DNase treatment has been shown to degrade and or destroy nucleic acid extracts, DNase treatment was not applied to RV papillary muscle specimens [21]. Reverse transcription (RT) was accomplished using Invitrogen's Super Script III cDNA synthesis kit, which is optimized for low inputs of RNA.

Quantitative PCR was performed using Applied Biosystem's (ABI) 7700 real time thermal cycler, ABI's Taqman Universal Master Mix with UNG, as well as ABI's pre-made primers and taqman probes for ANP, BNP, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a house keeper gene and its expression does not change with changes in experimental conditions [6, 21, 28]. GAPDH was used as an internal control. A Nanodrop spectrophotometer was used to measure RNA concentration. For each RNA sample, duplicate cycle threshold (Ct) values were obtained and averaged. The $\Delta\Delta$Ct method was applied to the averaged Ct values obtained, in order to quantify the relative changes in ANP or BNP gene expression [28].

2.3 Statistical Analysis

All data were reported as mean +/- SEM, with n representing the total number of samples. The standard error of the mean (SEM) describes the uncertainty of how the sample mean represents the population mean. Given n, the standard deviation, which describes the variability between individuals in a sample, can be calculated. Since cDNA concentrations are lognormally distributed and require nonparametric statistics, a two group t-test was performed on the ΔCt values [52]. Multiple linear regression as well as simple linear regression was utilized to compare the contribution of developed systolic stress, diastolic stress, and diastolic strain to the change in ANP gene expression. Significance was set at the $P < 0.05$ level.

Table 1: Time averaged electrolyte concentrations, gas partial pressures, and biocarbonate concentration of superfusate running through tissue culture chamber for 8 hour duration.

	Glucose (mg / dL)	Na^+ (mmol / L)	K^+ (mmol / L)	Ionized Ca^{2+} (mmol / L)	pH	PCO_2 (mmHg)	PO_2 (mmHg)	HCO_3 (mmol / L)
Superfusate:	95 +/- 1	163 +/- 2	5.4 +/- 0.1	1.45 +/- 0.02	7.46 +/- 0.02	24 +/- 1	469 +/- 30	18 +/- 1
Normal Blood:	100	145	5		7.35	35	100	25.5

3 Results

3.1 The Culture System

We have created a system to observe the development of hypertrophy in isolated intact cardiac tissue derived from mice. The muscle culture chamber (**figure 1**) enables stretching of mouse RV papillary muscles while simultaneously measuring muscle tension and local muscle deformation, within a biocompatible, physiological environment. A blood analysis system was utilized to monitor electrolyte concentrations, gas partial pressures, and biocarbonate concentration of the superfusate running through the system during the culture of papillary muscles for 8 hour duration. Values obtained were stable for 8 hour time period and comparable to those found in normal mouse blood [15] (**table 1**).

3.2 Mechanical Properties of Mouse RV Papillary Muscles

For the duration of the experiment twitch force and twitch kinetics were monitored to assess the viability of the tissue (**figure 2**). Peak isometric developed systolic tension was stable in most specimens (8 out of 10) for 5 hours of culture. Muscles were excluded from experimental groups if values in developed systolic tension declined by more than 50% and if diastolic tension increased by more than 50% at the five hour time point. Unlike specimens stretched to 10% of Lmax, larger fluctuations in developed systolic tension were evident in highly loaded specimens (**figure 2**), but degree of fluctuation was comparable to previously published results [6, 19] and had no effect on stretch induced ANP and BNP expression. Fluctuation in twitch kinetic parameters of highly loaded specimens was minimal over the duration of the experiment. Muscle stimulated at 0.5 Hz had a twitch duration of approximately 0.150 seconds and an average maximum developed systolic tension of 10.99+/- 2.22 kPa (n=9) (**figure 3**). Peak systolic stress was obtained at a lagrangian strain of $\sim$0.18, corresponding to an average diastolic stress of 18.42+/-2.64 kPa (n=4) (**figure 3**).

3.3 Effect of Mechanical Intervention on the Induction of Hypertrophic Markers

ANP and BNP gene expression is induced by increased mechanical loads in isolated wildtype cardiac myocytes as well as in whole heart preparations [33, 38, 46]. To determine the effect of mechanical load on the expression of ANP and BNP, gene expression was compared between muscles stretched to $\sim$90% or $\sim$10% of Lmax (control samples) using quantitative PCR techniques. The expression of the house keeping gene, GAPDH, was first analyzed between samples to determine if the gene is a stable internal control. GAPDH expression was found not to be effected by experimental treatment ($Ct_{90\%Lmax}$= 20.56 +/- 0.50; $Ct_{10\%Lmax}$= 20.99 +/- 0.45). Compared to controls, quantitative PCR results of BNP gene expression were 2.3 fold greater (P=0.02) in loaded specimens (**figure 4**) after two hours of stretch. A higher BNP expression was not evident at the 5 hour time point of stretch in loaded samples when compared to control samples. Due to the biphasic temporal induction of BNP, these results were not unexpected [50]. After 5 hours of culture, ANP gene expression was significantly higher in muscles stretched to 90% of Lmax (P=0.03) (**figure 4**).

A multiple linear regression model was computed for the normalized ANP gene expression as a function of developed systolic tension and di-

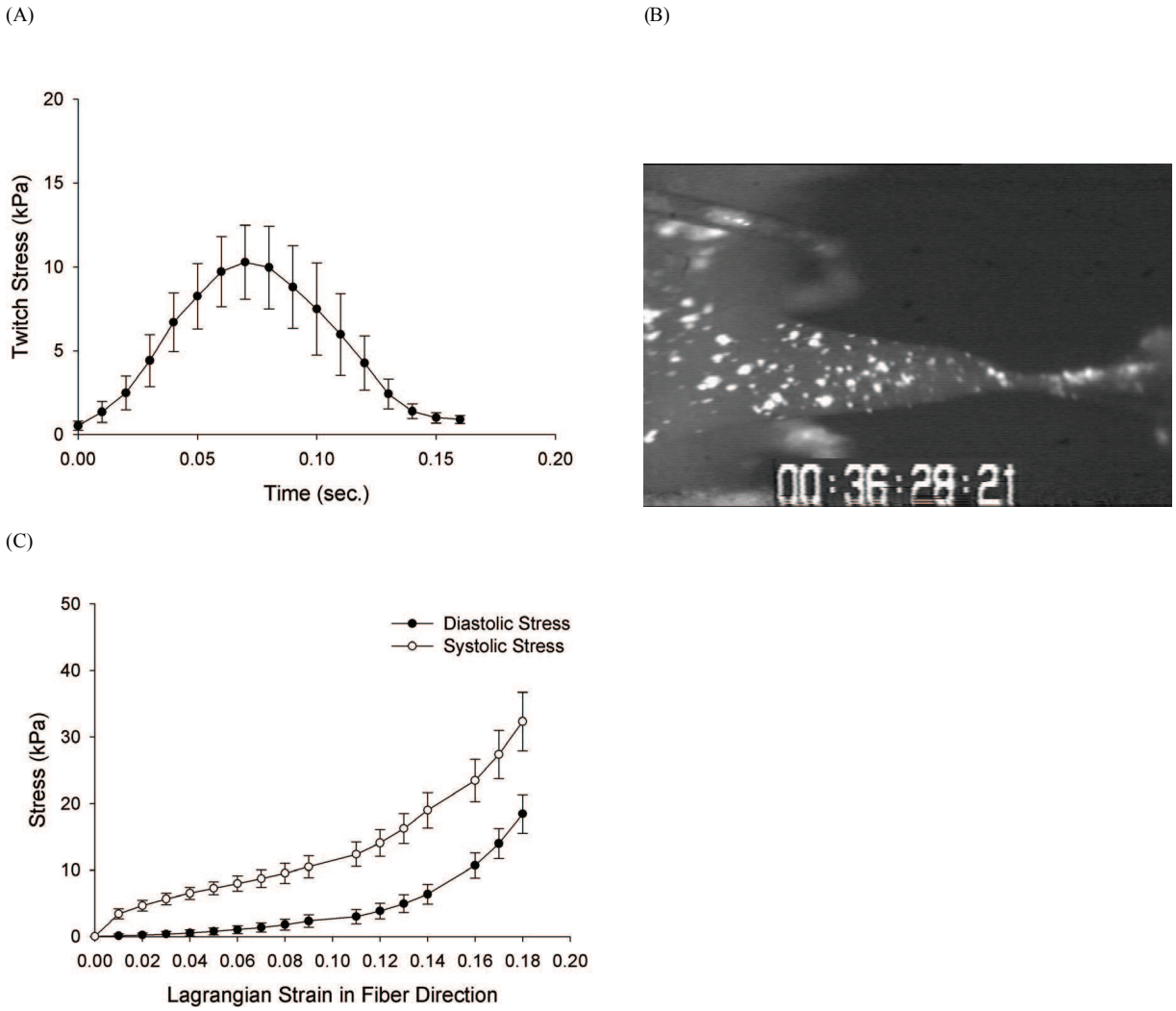


Figure 3: Mechanical properties of mouse RV papillary muscles. (**A**) Average twitch kinetics of mouse RV papillary muscles. (n=9) Data was normalized by unloaded cross sectional area. (**B**) Video image of mouse RV papillary muscle that has been marked with titanium dioxide in the loaded stretched state. (**C**) Average peak systolic stress strain relationship and diastolic stress strain relationship of mouse RV papillary muscles. (n=4) Data are mean +/- SEM.

astolic tension. The developed systolic tension plot exhibited more scatter than the diastolic tension plot (**figure 4**) indicating that ANP/GAPDH expression is not as highly correlated with developed systolic tension. Although the regression of the multiple regression model was shown to be significant (R^2=0.91, P=0.01), only diastolic stress was determined to contribute significantly (β=0.012, P=0.01) to the variability in ANP/GAPDH. A strong trend for the contribution of developed systolic stress (β=0.002, P=0.07) to the variability in ANP/GAPDH was shown by the model. The correlation between normalized ANP expression and diastolic stress (R^2=0.82, P=0.01) and normalized ANP expression and diastolic strain (R^2=0.84, P=0.01) was comparable.

To directly test if an increase in diastolic stress and/ or strain alone could explain the higher induction of ANP, electrically unstimulated muscles were stretched to 90% of Lmax for 5 hour duration. ANP gene expression was $\sim$2 times greater (P=0.04) in highly stretched electrically unstimulated muscles, when compared to electrically unstimulated samples stretched by 10% (**figure**

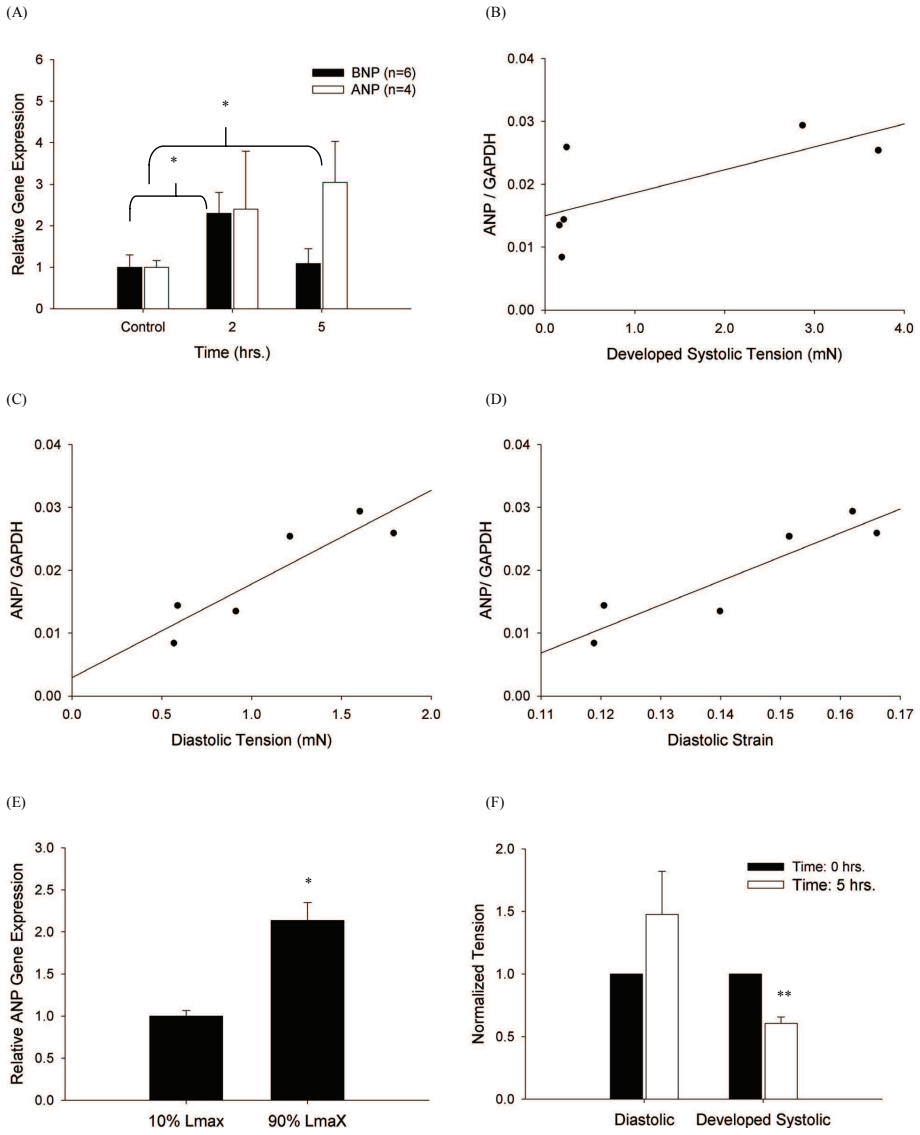


Figure 4: Increase in diastolic strain and/ or diastolic stress regulates natriuretic peptide gene induction. (A) Relative average gene expression data calculated from real time PCR results using the ΔΔCt. Muscles were stretched to 90% or 10% of Lmax and were induced to contract by electrical stimulation. Muscles were cultured for 2 or 5 hour duration. (B) Normalized ANP gene expression as a function of developed systolic tension. (n=6) (C) Normalized ANP gene expression as a function of diastolic tension. (n=6) (D) Normalized ANP gene expression as a function of diastolic strain. (n=6) Multiple linear regression model (R^2=0.91, P=0.01) was computed for ANP gene expression as a function of developed systolic (β_1=0.002, P=0.07) and diastolic tension (β_2=0.012, P=0.01). Simple linear regression models were computer for ANP as a function of diastolic strain (R^2=0.84, P=0.01) and ANP as a function of diastolic stress (R^2=0.82, P=0.01). (E) Relative average ANP gene expression of electrically unstimulated mouse RV papillary muscles. Muscles were stretched to 90% or 10% of Lmax. (n=3) Muscles were cultured for 5 hour duration. (F) Effect of sustained stretch (90% Lmax) on the developed systolic and diastolic tension of electrically unstimulated muscles. (n=3) * Indicates statistical significance (P<0.05). ** Indicates high statistical significance (P<0.005). Data are mean +/- SEM.

4). Peak isometric developed systolic tension in loaded electrically unstimulated samples declined significantly (P<0.005) at the 5 hours time point of stretch.

3.4 Mechanically Loaded MLPKO RV Papillary Muscles

Our previous study using cultured neonatal cardiomyocytes predicted that the mechanical load-dependent activation of selective hypertrophic responses is defective in MLPKO mice, a murine model of dilated cardiomyopathy [24]. Thus we compared the ANP gene expression in stretched papillary muscles obtained from MLPKO and control wildtype mice using the current culture chamber system. ANP gene expression in loaded muscles from MLPKO mice was ∼4 times lower (P=0.01) when compared to loaded wildtype muscles (**figure 5**). MLPKO muscles exhibited a significant decrease in developed systolic tension immediately after the initiation of culture (**figure 5**). At the five hour time point, however, an increase in developed systolic tension of MLPKO muscles was evident.

4 Discussion

4.1 System Characterization

In the present study, we have developed a mouse cardiac muscle culture system in which tissue deformation and or increase in tissue tension is utilized as a stimulus for the induction of hypertrophic gene expression. In contrast to isolated stretched neonatal ventricular myocytes, this system allows for the continuous monitoring of contractile forces, twitch kinetics, and stress strain relationship of isolated, adult, mouse cardiac muscle preparations. Developed systolic tension and twitch timing parameters of RV mouse papillary muscles cultivated in our system were preserved and stable for over five hours of culture, indicating the conditions of the system (**table 1**) as well as the diffusion of oxygen and nutrients to core of the muscle were sufficient to maintain tissue viability and contractility for the duration of the experiment. The critical diameter for optimal oxygen diffusion in rodent ventricular preparations in culture has been suggested to be about 0.20mm [43]. Preparations of thin mouse ventricular trabeculae would be preferable, however only a minority of mouse hearts have trabeculae suitable for isolation and culture. RV papillary muscles were chosen because they are thin (minor diameter: 0.24 +/- 0.03) and unbranched. Studies were not done on left ventricular papillary muscles because these muscles are thicker (minor diameter∼ 0.70mm) and diffusion of oxygen, substrates, and nutrients through the cross sectional area of these muscles may be inadequate. Additionally, ANP and BNP induction may be affected by a decreased oxygen supply in LV papillary muscles, consequently these specimens may not exhibit a normal stretch induced hypertrophic response when compared to control specimens [7, 39].

Maximum average initial developed systolic stress of RV papillary muscle preparations cultured in our system was 10.99+/- 2.22 kPa (n=9), which is comparable to other studies that have cultured mouse cardiac muscle under similar conditions of calcium concentration, temperature, and stimulation frequency [37, 42, 45]. Twitch duration, time from activation to peak contraction, and relaxation time were also comparable to other studies [19, 37, 42, 45]. Average developed systolic stress of wildtype muscles stretched to 10% of Lmax was stable for the duration of the experiment. Average developed systolic stress of wildtype muscles stretched to 90% of Lmax was more variable with time, but did not drop below 50% of the initial developed systolic stress. Contractile function of mechanically unloaded muscles left at slack length deteriorated with time. After a few hours in culture, mechanically unloaded muscles could no longer be induced to contract with electrical stimulation. In earlier studies, unloaded cat papillary muscles exhibit significant atrophy, marked increase in connective tissue, disorientation of contractile filaments, and loss of Z-line structure [9, 47]. It appears that cardiac tissue is extremely dependant upon mechanical loading, and loses baseline phenotype and normal contractile function in a very rapid timeframe if completely unloaded. For this reason, the expression of ANP and BNP of papillary muscles stretched to

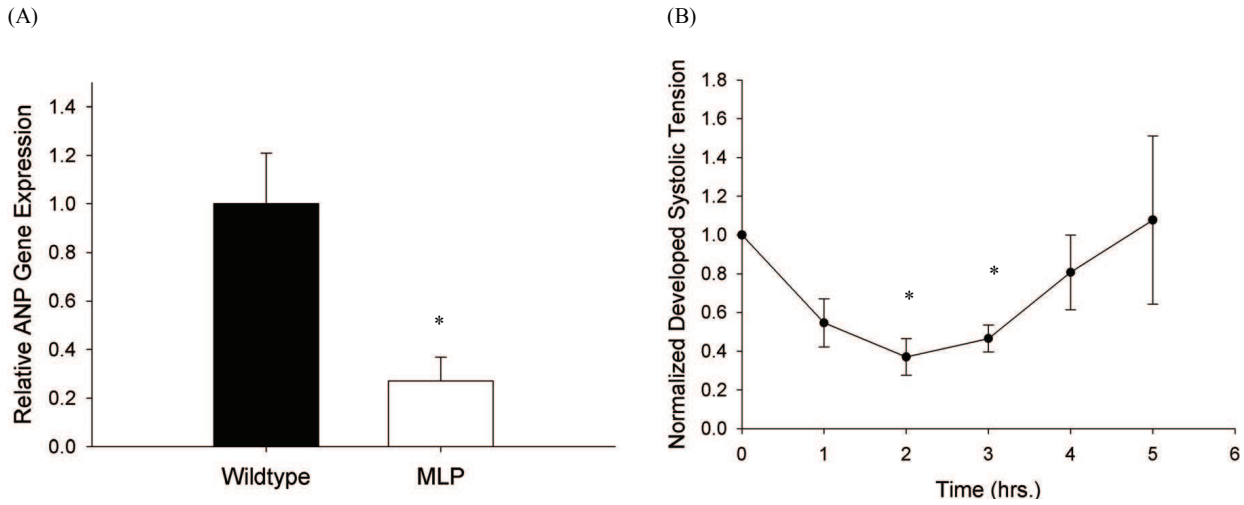


Figure 5: Chronic culture of MLPKO RV papillary muscles. (A) Relative average ANP gene expression data calculated from real time PCR results using the ΔΔCt. Muscles from two week old mice were stretched to 90% of Lmax and induced to contract by electrical stimulation. (n=3) (B) Average time course of systolic tension developed by MLPKO muscles. (n=3) Data was normalized by initial developed systolic tension. * Indicates statistical significance (P<0.05). Data are mean +/- SEM.

90% of Lmax was compared to muscles that were stretched to 10% of Lmax and not to muscles left at slack length.

Contractile function of cultured mouse cardiac muscle was also dependant upon electrical stimulation in our system. Developed systolic tension of electrically unstimulated muscles declined by 40% after five hours of culture. Diastolic tension was not affected by the absence of electrical stimulation. Previous studies have shown that electrical stimulation is essential for the preservation of contractile function of cultured adult cardiac myocytes [5]. It is therefore not surprising to find that the maintenance of contractile function of isolated cardiac muscle preparations was also dependant upon electrical stimulation. The decline in the ability of the muscle to develop systolic tension with electrical stimulation, however, had no affect on ANP induction.

4.2 Mechanical Load Induced Expression of Hypertrophic Markers

This study shows that a significant increase in gene expression of BNP and ANP, two clinically used markers of hypertrophy, occurred in response to an increase in systolic and diastolic mechanical load of isometrically contracting mouse cardiac tissue specimens. BNP gene expression was 2.3 +/- 0.5 fold greater in contracting, highly loaded samples after 2 hours of stretch. ANP gene expression was 3.0 +/- 1.0 fold greater in contracting, highly loaded samples after 5 hours of stretch. ANP gene expression is consistent with previous studies reported by Dr. Lang's group [20], who reported that ANP mRNA concentration in stretched left ventricular rat papillary muscles was about 3 times greater than unstretched controls. By developing a multiple linear regression model, we linked ANP expression in highly loaded specimens to higher diastolic loads (β=0.012, P=0.01), but not higher developed systolic loads (β=0.002, P=0.01). The highly correlative link between diastolic tension and diastolic strain prevented us from determining the relative contributions of stress and strain to ANP induction. Simple linear regression models revealed that these two regressors are significant (P=0.01). Individually each regressor explained about 80% of the variability (R^2=0.84, R^2=0.82) in normalized ANP expression, which is expected due to the high correlation that exists between stress and strain. ANP gene expression in electrically unstimulated samples stretched to 90% of Lmax was

2.1 +/- 0.2 fold higher than controls. This direct positive relationship between increased diastolic load and increased ANP expression suggests that diastolic stress and/ or strain is the predominant mechanical driving force for hypertrophic gene expression in isometrically contracting muscle specimens. Consequently, it may be the predominant mechanical stimulus for the development of hypertrophy and heart growth in vivo. Although the role of diastolic stress and or strain verses systolic stress and or strain in the development of hypertrophy is a controversial topic [34], recently evidence from other laboratories implies a greater role for diastolic mechanics in modulating myocyte remodeling of isolated cardiac tissues [17].

4.3 Effect of Load on the Expression of ANP and Systolic Tension of MLP null Myocardium

To determine if our methods have a suitable resolution and signal to noise ratio to detect differences in load induced hypertrophic gene expression between wildtype RV papillary muscles and muscles obtained from mouse hearts having defects in hypertrophic signaling, we stretched muscles from two week old Muscle LIM protein KO mice (MLPKO), a well characterized mouse model having significant diastolic dysfunction and dysregulated hypertrophic signaling [24, 29], and analyzed ANP gene expression in these specimens. Utilizing our techniques we were capable of measuring statistical differences in developed systolic tension of MLPKO muscles between the initial time and specific time points after the initiation of muscle stretch. As expected, the developed systolic tension of MLPKO cardiac muscles increased with an increase in mechanical load, but was not sustainable and decayed almost immediately following an increase in muscle length. This abrupt significant decrease in the contractile function of MLPKO muscles can be explained by multiple mechanisms such as a rapid decrease with time in the number of myofilament cross bridge interactions, in myofilament calcium sensitivity, and/ or intracellular calcium concentration [1]. Further investigation is required to identify the exact mechanisms involved, which was out of the scope of this study. ANP gene expression of highly loaded MLPKO specimens was also significantly lower than highly loaded wildtype muscles. This data confirms that the methods described in this paper are sufficient to analyze and study differences in load induced hypertrophic gene expression of isolated muscle preparations from mouse models of heart disease. It also demonstrates for the first time the use of mature cardiac myocytes from KO mice, instead of neonatal myocytes which have pronounced differences in phenotype when compared with adult myocytes, to study stretch induced changes in hypertrophic markers. Unlike previous results, which utilized an isolated neonatal ventricular myocyte stretch system [24], the current study provides direct evidence that load sensing and the normal hypertrophic response to an increase in mechanical load is blunted in MLP deficient mature cardiac tissue. In addition, it provides for additional insight into the mechanisms that lead to the onset of dilated cardiomyopathy evidenced in adult MLPKO mice.

4.4 Comparison with Current Multicellular Methods

Recently, at least two in vitro models of mechanical load induced hypertrophy, which have utilized excised cardiac muscle preparations, have been developed. In these studies rabbit RV trabeculae muscles as well as rat RV papillary muscles were cultured for over 35 hours [6, 17]. Although mouse RV papillary muscles cultured for 12 hours in our system exhibited a normal hypertrophic response (greater ANP protein expression, examined by immunohistochemistry) to higher mechanical loads and had preserved contractile function (Developed Systolic Tension=T, $T_{t=12hrs}$= (0.84 +/- 0.26)$T_{t=0hrs}$, n=4) for the duration of the experiment, only $\sim$10% of chronically loaded specimens survived for 12 hours in our culture chamber. Unlike adult rat cardiomyocytes, which can be cultured for multiple weeks [12], it is difficult to maintain viable adult mouse cardiomyocytes for long periods of time (>24 hrs) in culture [27]. Contractile function of mechani-

cally loaded rat RV papillary muscles cultured in our culture chamber was preserved for over 24 hours ($T_{t=25hrs}$=1.14$T_{t=0hrs}$). Due to the low success rate, we did not attempt to culture mouse RV papillary muscles for longer periods of time and assessed BNP and ANP gene expression at the 2 hour and 5 hour time points. Impressively, in these previous studies [6, 17], an increase in mechanical load was shown to have an effect on the size, shape, and protein expression of the tissue as well as the cardiac myocytes that compose it, directly illustrating evidence of cardiac hypertrophy. Although, in the limited time frame of 5 hours it is highly unlikely that we would see changes of cell size and shape of myocytes from RV mouse papillary muscles stretched in our system, we were still able to observe load induced changes in natriuretic peptide expression. In the clinical setting, the measurement of circulating natriuretic peptides is an established process for the diagnosis of left ventricular hypertrophy [32]. In our studies quantitative PCR techniques were used to analyze changes in mRNA concentration of atrial natrieretic peptide and brain natriuretic peptide. Quantitative PCR techniques were utilized because the amount of total RNA from a single mouse RV papillary muscle is too small ($\sim$ 200 ηg) to be visible on a standard gel. In myocardium and myocytes, increased expression of genes for natriuretic peptides is widely used as a marker of myocardial hypertrophy [13, 38]. Elevated levels of natriuretic peptide protein concentrations in blood plasma are correlated with an increase in natriuretic peptide gene expression, both of which correlate with changes in left ventricular size and shape [11, 52]. Although, these newly developed systems have the capability to analyze diastolic and systolic stress of cardiac tissue preparations [6, 17], they do not have the capability to evaluate local tissue deformation and consequently these studies failed to report the stress-strain relationship of the tissue.

The most pronounced difference between these newly developed systems [6, 17] and our own is the culture of cardiac tissue preparations from the murine species. The ability to mutate, delete, or overexpress selective genes in the murine heart has given scientists the possibility to identify genes that are causative for hypertrophy and heart failure. To date, several hundred genetically engineered mouse models of cardiovascular disease have been generated (http://tbase.jax.org) and are growing rapidly due to the availability of murine cardiac specific promoters. Only a few genetically engineered rat models and even fewer rabbit models have been developed. For this reason, the mouse has become the most studied model system. Consequently even though the culture of mouse cardiac tissue has certain limitations compared to systems using other species, the bulk of information available through the utilization of genetically engineered mice clearly has multiple uses in cardiac research.

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