Effects of increased preload on the force-frequency response and contractile kinetics in early stages of cardiac muscle hypertrophy

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Haizlip KM, Bupha-Intr T, Biesiadecki BJ, Janssen PM. Effects of increased preload on the force-frequency response and contractile kinetics in early stages of cardiac muscle hypertrophy. Am J Physiol Heart Circ Physiol 302: H2509–H2517, 2012. First published March 30, 2012; doi:10.1152/ajpheart.00660.2011.—Numerous studies have aimed to elucidate markers for the onset of decompensatory hypertrophy and heart failure in vivo and in vitro. Alterations in the force-frequency relationship are commonly used as markers for heart failure with a negative staircase being a hallmark of decompensated cardiac function. Here we aim to determine the functional and molecular alterations in the very early stages of compensatory hypertrophy through analysis of the force-frequency relationship, using a novel isolated muscle culture system that allows assessment of force-frequency relationship during the development of hypertrophy. New Zealand white male rabbit trabeculae excised from the right ventricular free wall were utilized for all experiments. Briefly, muscles held at constant preload and contracting isometrically were stimulated to contract in culture for 24 h, and in a subset up to 48 h. We found that, upon an increase in the preload and maintaining the muscles in culture for up to 24 h, there was an increase in baseline force produced by isolated trabeculae over time. This suggests a gradual compensatory response to the impact of increased preload. Temporal analysis of the force-frequency response during this progression revealed a significant blunting (at 12 h) and then reversal of the positive staircase as culture time increased (at 24 h). Phosphorylation analysis revealed a significant decrease in desmin and troponin (Tn)I phosphorylation from 12 to 24 h in culture. These results show that even very early on in the compensatory hypertrophy state, the force-frequency relationship is already affected. This effect on force-frequency relationship may, in addition to protein expression changes, be partially attributed to the alterations in myofilament protein phosphorylation.

contractility; culture; myofilaments; rabbit; trabeculae

HEART DISEASE IS THE NUMBER ONE killer in the western world, emphasizing the need for understanding the progression to failure and the development of different treatment strategies. There are numerous causes of heart failure, but a rather common denominator is the presence of some type of hypertrophic maladaptive growth of the myocardium. Cardiac hypertrophy is, most generally, caused by an increase in cardiac load, induced by elevated blood pressure (5, 31, 38). Normally, hypertrophy occurs as a compensatory mechanism to ameliorate the immediate effects of hypertension. This increased load, ultimately, leads to a deficiency in pump function of the heart. If this deficiency chronically remains, a hypertrophic response occurs, which is almost always irreversible.

Hypertrophy is characterized by an increase in the size of the cardiomyocyte, through the addition of sarcomeres in series and/or in parallel. The heart progresses through various phases of cellular growth on the way to failure; normal, compensatory hypertrophy, and decompensatory hypertrophy. After a stimulus triggers development of hypertrophy, initially, the heart responds with an increase in muscle force production, reduced myocardial stress, and improved cardiac function. This stage is referred to as compensatory hypertrophy. The increase in contractile strength, which is a characteristic of compensatory hypertrophy, can remain undetected for any number of years in a seemingly healthy individual (13). Here we attempt to investigate the functional and molecular alterations that occur during the hearts transition from the normal to the earliest phases of the compensated state.

There are several regulatory mechanisms that govern the contractility of the heart. These include the length-tension relationship, the β-adrenergic response, and the force-frequency relationship (1, 7, 12, 21). The force-frequency response (FFR) plays an essential role in adjusting the mechanical performance of the heart to the hemodynamic requirements of the body (12). In vitro and in vivo tests have shown that increasing the frequency at which the heart is paced leads to an increase in the contractile force (6, 26, 43). Heart failure is characterized by the decline of physiological regulators of cardiac contractility such as the FFR (12, 34). In larger mammals a positive FFR is associated with a healthy functioning heart, whereas a negative FFR is considered a marker of decompensatory hypertrophy or heart failure (17, 34, 37). Commonly, during clinical tests, exercise is used to determine a patient’s ability to increase their heart rate in response to mild exercise (39). Because compensatory hypertrophy does not initially manifest as pathologic, the relationship between frequency and force, before the compensated stage of hypertrophy, remains to be elucidated. Defining the contractile function of the myocardium during the various stages of development of compensatory hypertrophy could then possibly prove to be an early detection technique for those at high risk to develop heart failure.

To elucidate if the FFR could be a significant indicator in our system for the progression to heart failure, we investigated the effects on the FFR during the earliest development in progression to compensatory hypertrophy in a high preload isolated muscle culture system. This novel in vitro hypertrophy system has, previously, allowed our laboratory to study the effect of high preload on remodeling and contractile failure (9). Muscles that have been cultured for 48 h transition from normal, to compensatory, to decompensated states of hypertrophy. Cultured muscles are able to be visualized, in real-time, and the effects of stretch on the increase in muscle growth can be measured (9, 25). Based on our previous studies, we also aim to elucidate the molecular mechanism of functional alterations in FFR during cardiac remodeling. Molecular alterations will

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be determined through the analysis of changes in phosphorylation state of myofilament proteins.

MATERIALS AND METHODS

The present study conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). All of the experiments were conducted under protocols approved by the Laboratory Animal Care and Use Committee of the Ohio State University.

Multicellular myocardial culture. The cardiac trabeculae culture procedure has been detailed previously (25). Briefly, New Zealand white rabbits (1.5–2.0 kg) were heparinized, and anesthetized, by ear vein injection of pentobarbital sodium (50 mg/kg). The hearts were rapidly excised and retrogradely perfused in a modified Langendorf perfusion system with a 2,3-butanedione monoxime-containing low calcium Krebs-Henseleit solution. Thin, nonbranched trabeculae, from the free wall of the right ventricle, were dissected and then mounted between the force transducer and a micromanipulator screw, in a semi-closed circuit culture system (9). Previous studies have shown that protein synthesis and turnover is maintained (25) and that this system can deliver stable contractile function for as long as 5 full days (24). Right ventricular muscles were used to aid in comparison from previous studies conducted by us and others. Additionally, the abundance of trabeculae in the right ventricle enhances experimental efficiency. Left ventricular trabeculae are typically branched, or shorter, and cannot unambiguously be used for linear assessment of force in vitro. The solution was exchanged and the muscles were subjected to isometric contractions by stretching to a force in vitro. The solution was exchanged and the muscles were shorter, and cannot unambiguously be used for linear assessment of efficiency. Left ventricular trabeculae are typically branched, or shorter, and cannot unambiguously be used for linear assessment of force in vitro.

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RESULTS

Alternations in muscle dimension induced by culture at high preload. Figure 1 demonstrates no change in muscle dimensions during the 12- and 24-h culture period at high preload. It also demonstrates that when the exposure to high preload was extended to 48 h, marked increases in dimensions were observed.

Contractile function in response to mechanical overload. Isometric contractions at a high preload for 24 h leads to a mild increase in developed force over baseline recordings (taken after 2 h of stabilization into culture period). Pacing at 1 Hz over a 24-h period leads to an increase in force production (Fig. 2A). This suggests that there is significant muscle strength and viability to conduct a FFR test at all times leading up to, and including, the 24-h mark. Further analysis of additional contractile parameters reveals an increase in time-to-peak tension and time from peak tension to 50% and 90% relaxation (RT50 and RT90, respectively; Fig. 2, B–D). As expected with increases in force development the time required for the twitch to complete is also extended as apparent in the increase in time-to-peak force development and relaxation. In a set of n = 6 muscles, under very low-preload conditions, changes in force development were virtually absent, as were any changes in twitch kinetics. The lack of no significant preload, however, precluded direct measurement of changes in force during changes in frequency, as at increased frequency the reduced diastolic tension completely unloaded the muscle, prohibiting accurate assessment of force and kinetics. Figure 3A shows that with increasing culture time there is no effect on contractile force. Additionally, time from stimulation to peak tension (TTP), RT50, and RT90 (Fig. 3, B–D) remain, relatively, stable, and lower than values recorded during high preload culture.

Effects of culture time on force frequency response. Initially, we aimed to determine the effects for preload and remodeling...
on FFR and hypothesized that with an increase in force at baseline there will be an altered FFR. Upon stimulation for 24 h we discovered a gradual decline in the positive staircase of the FFR (Fig. 4A). This suggests that although the contractile force of the muscle is increased at 24 h compared with baseline, there is a decrease in contractile reserve (i.e., the ability of the myocardium to increase its contractile force upon increases in frequency) that occurs in response to remodeling that blunts and eventually eliminates the positive FFR, which is indicative of a decline in cardiac reserve. Maximal twitch force attained by the muscles is on average $22 \text{ mN/mm}^2$ (4 Hz at 0 h), $20 \text{ mN/mm}^2$ (3 Hz at 12 h), and $20 \text{ mN/mm}^2$ (1 Hz at 24 h), which indicates a shift in frequency-dependent behavior, but not necessarily a loss of maximal twitch force. Tracings normalized to 1 Hz force development at each time point show a gradual blunting of force with increasing frequency as culture time increases (Fig. 4B). Normalized change in force tracings from 1 to 4 Hz recapitulate the negative FFR at 24 h as compared with 0 and 12 h.

Effects of culture time on regulatory contractile kinetics during the FFR. Analysis of the kinetics during the FFR showed an acceleration of time-to-peak force production with increasing frequency at all time points, as expected (Fig. 5A). Change in TTP from 1 to 4 Hz at 12 and 24 h is, significantly, increased from baseline; this reinforces the decrease in cardiac reserve that is seen to occur in progression to failure (36). The changes of contractile kinetics mostly carry over to relaxation rate (Fig. 5, C and D) where acceleration of relaxation rate to 50% and 90% occurs at most frequency steps.

Load and time-dependent alterations in phosphorylation state of myofilament proteins. Increases in troponin complex proteins, myosin heavy chain, and myosin light chain (MLC) have been known to occur in hypertrophy and heart failure (11); however, understanding exactly which changes are associated with re-
modeling and compensated versus decompensated failure remains to be determined. Current models of in vivo trans-aortic constriction and left-anterior descending artery banding have revealed multiple contradictory findings, and myocyte studies have been inconclusive due to cellular viability (4). Here we aimed to determine the effects of remodeling on the post-translational modifications of contractile proteins thought to govern cardiac contractility. Analysis of key contractile proteins through phospho-protein analysis reveals an increase in phosphorylation of TnI, TnT, MLC-2, desmin, and MyBPC at 12 h as compared with 0 and 24 h (Fig. 6A). Phosphorylation of TnI and desmin significantly decreases from 12 to 24 h after culture to levels below basal values (0 h culture) (Fig. 6B). These findings suggest a regulatory mechanism of phosphorylation that first increases contractile function at 12 h, by maintaining the FFR. This increase in function is then lost during the decline in overall phosphorylation at 24 h.

DISCUSSION

Chronic stretch, or persistent increases in the mechanical load that the heart must pump against, are one of the main events leading to heart failure. In this study we aimed to determine the effects of stretch, in long term culture, on alterations to the FFR. Through the use of a recently developed in vitro muscle hypertrophy system (9, 15), we were able to determine that 1) stretch/high preload induces a process eventually leading to an increase in muscle size and contractile strength, 2) the sustained increase in preload leads to a transient increase in phosphorylated myofilament proteins, and 3) the FFR gradually becomes blunted and eventually negative as culture time increases from 0 to 24 h. These results are taken in conjunction with our numerous previous studies that highlight the capabilities of the in vitro model system. We have shown previously that with our system we are able to maintain protein transcription and the response to physiological regulators (25), induce myocyte growth over time (9), and observe load-dependent alterations in protein expression (8, 9). To advance our understanding of the alterations on the FFR in this model, we observe contractile characteristics to determine the maintenance of muscle health and visualize the overall response of preload over time. Previous studies have shown that this in vitro hypertrophy is due to sarcomere addition and not
to edema (9, 25), and when the muscle is kept at low or moderate preload, contractile parameters, including the force-frequency relation, and protein composition/turnover, are stable over time (15, 23, 25, 27, 28, 30). The current data suggest that we are able to reproduce a high preload-induced cardiac growth response in vitro. In this study, we set out to quantify functional changes that would occur as the muscle shifts from a healthy toward a hypertrophic state. An assessment of force-frequency changes in already hypertrophied muscle (such as pulmonary artery banded rabbits in which the force-frequency relationship is already blunted) (14, 44) would allow us to potentially follow the transition to end-stage failure, but this was deemed well beyond the scope of the current study.

The effects of chronic stretch-induced pressure and volume overload on the heart have been well documented (2, 9, 33). The Frank-Starling law of the heart dictates that with increasing preload comes increasing developed force production (41). This is partially due to altered myofilament properties leading to an increase in affinity for calcium of TnC and causes an increase in cross-bridge formation (10, 40). The observed muscle sizes with prolonged preload and changes in contractile force suggest that sustained increased preload leads to an increase in developed force. This effect is regardless of changes in muscle size, and similar to our previous report (9). In our current study, we aimed to determine alterations in contractile function that could serve as a marker for the early compensated state. We were able to show enhanced contractile kinetics recorded as increases in the time-to-peak force production, and rate of relaxation to 50% and 90%, and an increase in basal contractile force (Fig. 2). Under basal stimulation in the time-to-peak force production, which can be substituted for the rate of shortening, there is a trending increase. Upon stimulation at higher frequencies, increases in culture time lead to an increase in rate of shortening (Fig. 5B), which is indicative of slower kinetics. This suggests an innate enhancement of contractile function that occurs solely due to high preload (or stretch) induced at basal levels.

The Bowditch Effect, or FFR, is commonly used to determine the viability of cardiac muscle (6). Normally, when healthy myocardium is paced, at gradually increasing frequencies, there is an increase in the contractile force produced by that muscle (26). Previous studies have shown a decreased force capacity when beat frequency is increased in failing muscles, as compared with nonfailing muscles (19). A negative FFR is thought to reflect a decrease in sarcoplasmic reticulum calcium load (19, 37). In addition to altered calcium handling, the FFR also appears to be regulated at the myofilament level: upon an increase in stimulation frequency, myofilament calcium sensitivity has been shown to be reduced in the rabbit (43), and this myofilament contribution can become dysfunctional under pathological conditions (44).

In our particular system a normal, blunted, and negative FFR can be detected during different times in culture. At 0 h, we observe the typical positive frequency staircase. At 12 h, this positive staircase is now slightly blunted, yet remains positive. At 24 h, the FFR has become significantly negative. In vitro studies have shown that the force generating capacity of failing muscles is only seen to decline with increased frequencies (19). Experimentally, we are able to better determine the point in which the muscle transitions from healthy (time = 0 h), to compensating (time = 12 h), to a failing (time = 24 h) model. Although a negative FFR is often seen during end-stage failure, this study shows that changes in the FFR can be detected before our experimental failure model and seems to be initiated, and manifest, well before an increase in basal contractile force, and well before the actual developing hypertrophy.

Decreasing preload generally leads to the reverse remodeling of cardiac hypertrophy. In our present study, we aimed to determine the role of sustained presence of preload on FFR. Figure 3 shows the analysis of low/no preload muscles. Upon culturing for 24 h at low preload, there is no significant effect on developed force, TTP, or relaxation time. Experiments conducted on FFR, on low preload muscles, show no change in contractile function induced by time. However, because at low load the FFR is negative, data analysis is not reliable for these experiments. Additionally, due to the noise level of muscles contracting at low preload, analysis of twitch kinetics was deemed impossible. Contractile maintenance, at normal pre-
load, has been recapitulated in previous studies during culture periods that surpass our current study (24).

Phospho-protein analysis revealed that from 0 to 12 h, there is an increase in phosphorylation state of key contractile proteins including TnT, MLC-2, MyBP-C, desmin, and TnI. This increase in phosphorylation is followed by a decline from 12 to 24 h. Through the use of ProQ diamond and Lava Purple total protein staining, significant decreases in TnI and desmin phosphorylation were recorded from 12 to 24 h specifically. Additional studies on the phosphorylation state of TnI during heart failure, which is commonly named as a major culprit in altered contractile kinetics, show a decrease in phosphorylation level during failure (29, 35). A common marker for heart failure, and therefore remodeling, are alterations in calcium handling (16). Our studies on the FFR suggest a mishandling of calcium through either the myofilament-calcium interaction or calcium transients (43). Previous studies in our laboratory, with the use of the calcium indicator dye fura-2, have revealed decreases in myofilament calcium sensitivity with increased frequency. It is thought that the decline in myofilament calcium sensitivity occurs in response to an increase in TnI phosphorylation (43). Other studies have highlighted remodeling induced decreases in connexin 43, decreasing the ability for cell-to-cell communication (8). Alterations in calcium sensitivity may explain the effects seen with increasing frequency and changes in the rate of time-to-peak force production. Here we show that changes in baseline phosphorylation state occur in the in vitro culture model. This change in phosphorylation occurs in conjunction to the decreasing capability of the muscle to increase in contractile force with increasing frequency; the phosphorylation state of TnI is also decreased.

Desmin is a complex component that helps to maintain the structural integrity and contractile apparatus of the functioning myocyte (18, 36). Increasing amounts of desmin, and alterations on the intermediate filament, have been shown to occur in rat myocytes after mechanical load-induced hypertrophy (45).
Prior studies have indicated that although desmin is able to be phosphorylated, detection methods lack the sensitivity to determine qualitative changes in phosphorylation state in human cardiac biopsies (20, 46). To what extent phosphorylation of desmin plays a direct or indirect role could not be determined. Interestingly, at 12 h, there is an increase in desmin phosphorylation, possibly suggesting the involvement of the progression of the hypertrophic event via intermediate filament disassembly (20). The results of this study indicate involvement of phosphorylation pattern changes in the myofilament matrix proteins and allow us to direct future investigations into the involvement of specific phosphorylation sites.

All the muscles in this study, regardless of preload level, contracted isometrically, so in principle with infinite afterload. However, internal elastance and sarcomere inhomogeneity permits some central segment shortening, which occurs less at higher preload, as we have shown in the past using laser-diffraction studies (22). As such, an unambiguous differentiation between pre- and afterload cannot be made, and the preload settings may induce an increased functional afterload that could be at the basis of the impaired FFR as culture time progresses and the muscles move toward hypertrophy.

In conclusion, mechanical loading plays a key role in the progression to a compensated hypertrophic state. In our particular model system the earliest stages leading to compensatory hypertrophy are characterized by an increase in overall developed force along with a decrease in rate of contractile kinetics such as TTP and RT50. We have also shown that the Bowditch Effect is slowly being reversed during the progression to compensatory hypertrophy in our in vitro model. This reversal occurs well before histologically detectable changes in cell size, which occurs later in the progression of dysfunction.

Fig. 6. Immunoblot analysis of phosphorylated contractile proteins at 0 (n = 4), 12 (n = 4), and 24 (n = 4) h high preload. A: Lava purple total protein stain of analyzed bands MyBPc, desmin, actin, troponin I, and myosin light chain-2. MW, molecular weight. B: phosphoprotein (Pro-Q Diamond) stain of aforementioned bands for analysis. C: ratio of band density of phosphoprotein to actin increases in desmin at 12 h and decreases in desmin and TnI at 24 h, as compared with 0-h values. D: change in phosphorylation state normalized to 0 h of myofilament proteins. Desmin increases ∼2.5-fold over baseline levels at 12 h. Desmin and TnI decrease 0.5-fold at 24 h as compared with baseline values. *Significance as compared with 0 h; **significance as compared with 12-h.
in this model. This alteration in FFR could be coupled with the alterations in basal phosphorylation state of myofilament proteins (TnI and desmin), which may act through a compensatory mechanism early on, and lost later at the peak of the compensated state. Further in-depth studies to reveal temporal changes in alterations on calcium handling induced by remodeling are needed to better determine effects on the calcium-handling component of the FFR and frequency-dependent acceleration of relaxation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.M.H. and P.M.J. conception and design of research; K.M.H., T.B.-I., and B.J.B. performed experiments; K.M.H., B.J.B., and P.M.J. analyzed data; K.M.H. and P.M.J. edited and revised manuscript; K.M.H., T.B.-I., and B.J.B. interpreted results of experiments; K.M.H. and P.M.J. prepared figures; K.M.H. drafted manuscript; K.M.H. and P.M.J. edited and revised manuscript; K.M.H., T.B.-I., B.J.B., and P.M.J. approved final version of manuscript.

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