Published in final edited form as:

J Mol Cell Cardiol. 2012 August ; 53(2): 282–290. doi:10.1016/j.yjmcc.2012.04.016.

Automated imaging reveals a concentration dependent delay in reversibility of cardiac myocyte hypertrophy

Karen A. Ryall¹ and Jeffrey J. Saucerman^{1,*}

¹Department of Biomedical Engineering, University of Virginia

Abstract

Cardiac hypertrophy is controlled by a dense signaling network with many pathways associated with cardiac myocyte growth. New large scale methodology is required to quantitatively characterize the pathways that distinguish reversible forms of hypertrophy from irreversible forms that lead to heart failure. Our automated image acquisition method records 5×5 mosaic images of fluorescent protein-labeled cardiac myocytes within each well of a 96-well plate using an automated stage and focus. Postprocessing algorithms automatically identify cell edges, quantify cell phenotypes, and track cells. We uniquely applied our imaging platform to study hypertrophy reversibility in a scalable cell model. Cell area changes after washout of a dose response to the α -adrenergic receptor (α AR) agonist phenylephrine (PE) showed that hypertrophy reverses at low but not high levels of α -adrenergic signaling: a reversibility delay. Perturbations with specialized α AR antagonists, a mathematical model, and live imaging of α AR localization identify the mechanism for this reversibility delay: ligand trapping with internalized PE acting on intracellular α AR's.

Keywords

cardiac hypertrophy; automated imaging; α -adrenergic signaling; cardiac myocytes

1. Introduction

Cardiac hypertrophy develops in response to stresses on the heart as the body attempts to compensate for reduced cardiac output. While this response may be adaptive initially, if stresses persist, an apparently irreversible decompensation can occur leading to heart failure [1]. A dense signaling network manages this response, with numerous pathways implicated in the hypertrophy phenotype [2]. A quantitative understanding of the pathways and their interactions is needed to understand how context dependent decisions are made by myocytes to control heart growth and in order to develop more effective therapies for heart failure.

Reversing hypertrophic remodeling remains a key objective for advancing therapies for heart failure. Nearly every successful clinical treatment for heart failure associated with improvements in long-term clinical outcomes reverses remodeling [3]. Despite this, little is

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author: Jeffrey J. Saucerman, Ph.D., PO Box 800759, University of Virginia, Charlottesville, VA 22908-0759, USA, Phone: 434-924-5095, Fax: 434-982-3870, jsaucerman@virginia.edu.

Disclosures: none declared

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

known about the specific signaling pathways that distinguish reversible forms of hypertrophy from irreversible forms which lead to heart failure. Few comprehensive studies of hypertrophy reversibility have been conducted and none in a cell culture model. Previous studies have shown reverse remodeling in mouse models [4–6] and in patients after medical device implantation or other surgical interventions [7–9]. While these studies support the idea that targeting reversibility of pathological hypertrophy may be a viable therapeutic strategy, studying reversibility of hypertrophy in a cell model will be essential for systematically exploring reversibility mechanisms and screening for potential drug targets.

Current approaches to study hypertrophic signaling have been low-throughput and qualitative, probing isolated pathways. These studies are useful in determining a functional role of a gene or protein, but experiments providing quantitative, temporal, and spatial information are needed to fully understand network organization in a complex process like cardiac hypertrophy [10]. This will require new large scale experimental approaches that can measure a large number of input and output relationships at multiple time points [11]. One promising approach is high-content cell imaging, which can quickly and reproducibly generate high volumes of quantitative data across spatial and temporal dimensions [12]. Recently automated segmentation has been used to study cardiac hypertrophy, but only in fixed cells [13], [14].

Here, we developed a high-throughput screening procedure to study the hypertrophy signaling network in live cardiac myocytes. Using automated large-scale microscopy and image analysis, thousands of individual cardiac myocytes were measured and tracked over several days. This new platform was applied to study reversibility kinetics of phenylephrine (PE)-induced cardiac myocyte hypertrophy. Our approach revealed that PE-induced hypertrophy exhibits a concentration-dependent reversibility delay, with sustained hypertrophy after agonist washout in myocytes exposed to high initial concentrations of PE. Additional perturbations and a mathematical model demonstrate that this reversibility delay can be mechanistically explained by internalized PE acting on intracellular α -adrenergic receptors.

2. Materials and methods

2.1 Cell culture

Cardiac myocytes were harvested from 1–2 day old Sprague Dawley rats using the Neomys isolation kit (Cellutron, Baltimore, MD). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the University of Virginia Institutional Animal Care and Use Committee. Myocytes were cultured in plating media (Dulbecco Modified Eagle Media, 17% M199, 10% Horse Serum, 5% Fetal Bovine Serum, 100U/mL penicillin, and 50 mg/mL streptomycin) on Cellbind treated 96-well plates (Corning, Corning, NY) at a density of 100,000 cells/well. Two days after isolation, myocytes were transfected with GFP driven under a cardiac myocyte specific troponin T promoter [15] using Lipofectamine 2000 (Invitrogen, Carlsbad, California; transfection efficiency: 10–15%; Supplementary Fig. S1).

Two days after transfection, myocytes were imaged using an Olympus IX81 inverted microscope with 10X UPlanFLN 0.30 NA objective, Orca-AG CCD camera (Hamamatsu, Bridgewater, NJ), automated stage (Prior Scientific, Rockland, MA), and IPLab software (Scanalytics, Fairfax, VA). Images were acquired with 120 ms exposure time using a 480/40-nm excitation filter and 535/50-nm emission filter (Chroma filters; Optical Insights, Santa Fe, NM). After imaging, cells were rinsed and transferred to serum-free media (Dulbecco Modified Eagle Media, 19% M199, 1% ITSS, 100U/mL penicillin, and 50 mg/mL streptomycin) with a given concentration of PE, an α -adrenergic receptor (α AR)

agonist. After 24 hours, cells were imaged, rinsed twice, and cultured in serum-free media without PE. Follow-up images were recorded every 24 hours.

2.2 Automated microscopy

Software algorithms were developed to automatically focus, capture a 5×5 grid of images in each well of interest in a 96-well plate, and assemble these images into a composite mosaic image. This allows for a highly reproducible imaging protocol in which the same region of cells can be imaged and tracked over the entire four day imaging period. Mosaic images enhance the number of cells imaged per well. The 5×5 mosaic encompasses an approximately 0.06 cm² region, which is about 20% of the cell growth area of a well and contains ~150 GFP-expressing cardiac myocytes. In contrast, a single image captures less than 1% of the cell growth area of a well and on average contains less than 10 myocytes.

Using centroid coordinates for a 96-well plate, the imaging software directed the motion of the stage to the center of each well in a left to right serpentine motion (Fig. 1A). Within each well, an autofocus operation was performed by positioning the objective to five different heights, 50 μm apart, and recording an image. The z-step resulting in the highest contrast image was selected and then the process was repeated at five smaller z-step intervals 2 μm apart. The objective height resulting in the highest contrast image of the 2 μm z-steps was the focus used for image collection in that well.

The automated stage then moved to each position in a 5×5 grid about the centroid of the well in a left to right serpentine motion, capturing an image at each location. This grid of images was then assembled into a composite mosaic image (Fig. 1A) and saved with a filename labeling the coordinates of the well on the 96-well plate. This process was repeated for every well of interest on the plate. Imaging for these experiments lasted 10–20 minutes and cells were kept in an incubator between imaging periods.

2.3 Automated image analysis

Based on the filename, the algorithm first loads the four 5×5 mosaic images saved in 16-bit Tiff format of the same well imaged on four different days. A threshold of 0.01 is set to reduce background noise and the intensities are rescaled to a range from 0 to 1, which is a requirement for the segmentation step in the algorithm. Next, a pixel intensity that distinguishes objects from background is found using the Otsu method [16].

An advantage of this approach is that given the transfection efficiency (10–15%) using Lipofectamine, expressing cells are rarely adjacent to each other, allowing for conspicuous cell boundaries. However, in the event of a cluster of expressing myocytes, cells are differentiated by intensity value, since GFP expression tends to be higher in the center of the cell. The number of cells in a group is determined by counting the number of local maxima of intensity in a smoothed image. Then dividing lines between adjacent cells are calculated with a watershed algorithm [17] using the previously identified local maxima of intensity as starting points. Any objects touching the border of the image are discarded.

Using the identified cell boundaries (Fig. 1B), various metrics of cell shape are calculated for each cell including area, perimeter, eccentricity, major axis length, minor axis length, form factor, and orientation. Here, the output of interest was cell area. We validated the use of myocyte area as representative of three-dimensional cell growth for PE-induced cardiac myocyte hypertrophy (Supplementary Fig. S2). After segmenting the first image of the four day sequence, each cell is given an ID number and tracked between subsequent images based on distance the closest cell in the next image to its original position is labeled with the same ID number. The tracking algorithm searches a neighborhood of 100 pixels surrounding the previous location of each identified myocyte. Since cardiac myocytes migrate minimally,

the myocytes stayed in the same location between subsequent images and distance was a robust method of tracking the myocytes (Fig. 1C). The ID number of each cell is saved in the output file, so that the measurements can be sorted in post-processing. The tracking step in this algorithm therefore allows for changes in measurements of individual myocytes to be observed over time. Fold change data output using the tracking algorithm have less dispersion and a more peaked histogram than the raw area data collected without tracking (Supplementary Fig. S3).

The automated cell segmentation and tracking algorithm was implemented using the open-source MATLAB-based CellProfiler software package [18]. Matlab scripts were developed to sort area measurements based on the saved cell ID numbers. Only myocytes with an area measurement for all four days were included in our data analysis. All algorithms are freely available for download at <http://bme.virginia.edu/saucerman>.

2.4 BODIPY-prazosin competitive inhibition experiments

Cardiac myocytes were given a 10 nmol/L solution of BODIPY-FL-prazosin (Molecular Probes) in serum free media for five minutes and then imaged using a Zeiss LSM 510 META laser scanning microscope with a 63X oil Plan Apochromat 1.4 NA objective, LSM 4.0 META software, and a 40 mW Argon-ion laser generating the 488-nm line. 3D stacks were taken for a subset of the imaged myocytes at 0.5 μm z-steps. For competitive inhibition experiments, myocytes were preincubated with a solution of 10 $\mu\text{mol/L}$ phentolamine, 1 mmol/L PE, or 100 $\mu\text{mol/L}$ CGP-12177a in serum free media for ten minutes before adding BODIPY-prazosin, giving a final concentration of 10 nmol/L BODIPY-prazosin. Images were collected after myocytes were in solution with BODIPY-prazosin for five minutes. BODIPY-prazosin labeling was quantified by segmenting the imaged myocytes using the phase-contrast channel and then calculating the above threshold integrated intensity of the BODIPY-prazosin channel normalized by cell area for each myocyte imaged. The threshold was set as the pixel intensity three times above the mean background intensity in the BODIPY-prazosin channel. The median and interquartile range were calculated for each condition. Differences in above threshold integrated intensity/cell area were tested for statistical significance using Kruskal-Wallis non-parametric one-way analysis of variance followed by a Dunn's multiple comparisons post-test.

3. Results

3.1 Concentration dependent reversibility of PE-induced hypertrophy

This automated imaging platform provides a unique opportunity to examine the reversibility of cardiac myocyte hypertrophy. Previous cellular studies have primarily examined single time points, preventing the observation of dynamics of cell growth generated by various hypertrophic agonists. Since this platform can track morphological changes in individual cardiac myocytes, mechanistic studies of hypertrophy reversibility are possible. Here, we studied the reversibility of cell growth after application of several different concentrations of PE.

After recording initial images (day 0), the cardiac myocytes were cultured in serum-free media containing varying PE concentration for 24 hours and then rinsed and switched to serum-free media without PE. Images were collected every 24 hours. On day 1, myocytes exhibited concentration-dependent increases in cell area, as expected, highlighting this platform's ability to quantify distinct levels of cell growth (Fig. 2A). Levels of PE-induced hypertrophy are consistent with the range reported in the literature using other imaging methods [13], [19], [20]. After PE washout, myocytes exposed to lower concentrations (PE 10 $\mu\text{mol/L}$) began decreasing in size, reverting to approximately their original area within 48 hours after washout. Conversely, myocytes exposed to the highest concentrations

(PE 100 $\mu\text{mol/L}$), continued to increase in area after agonist washout, with the 1 mmol/L condition showing increasing fold changes in cell area throughout the entire 72-hour data collection period. Fig. 2B shows representative cells exposed to 1 $\mu\text{mol/L}$ or 1 mmol/L PE.

Differences in fold change in cell area on day 1 and day 3 were tested for statistical significance using Kruskal-Wallis non-parametric one-way analysis of variance followed by a Dunn's multiple comparisons post-test test (Supplementary Table S1). To confirm that this finding was not the result of residual PE after washout in the high concentration conditions, we also measured cell growth in myocytes exposed to PE for 30-second intervals (Supplementary Fig. S4).

Integrated fluorescence intensity of the GFP driven by the TnT promoter follows a similar pattern as cell area after exposure to PE (Supplementary Fig. S5). Myocytes exposed to the highest concentrations of PE continue to increase in integrated GFP fluorescence after agonist washout at 24 hours, while the lower concentrations begin to decrease in integrated intensity after agonist washout. We performed additional validations to ensure that increases in integrated GFP fluorescence upon application of PE were not affecting myocyte area measurements (Supplementary Fig. S6). Integrated GFP fluorescence would not increase if the cells were only spreading out. Thus the integrated GFP fluorescence intensity provides a reporter of troponin T promoter activity that is relatively independent of myocyte growth.

The data show that the kinetics of cardiac myocyte hypertrophy after removal of PE is a concentration dependent reversibility delay: PE-induced hypertrophy is reversible at low concentrations of PE and persists at higher concentrations despite washout of the agonist. This surprising result has implications for a memory response in αAR -mediated hypertrophy. It suggests that hypertrophy may persist for a significant time period after exposure to transient circulating αAR agonists resulting from the neurohumoral response to stress. This memory response could accelerate development of hypertrophy and hinder its reversal.

3.2 Evidence for the role of intracellular αAR 's in reversibility delay

We hypothesized that sustained αAR signaling inside the cell allowed for the continued increase in cell area even after extracellular washout of PE. This could be mediated by cellular uptake of PE. Therefore myocytes given high extracellular concentrations of PE could potentially transport enough PE into the cell during the 24 hour exposure period for sustained increases in cell area. Recent evidence that αAR 's colocalize with intracellular endosomes [21] and the nuclear membrane in adult cardiac myocytes [22] provides a potential mechanism for this intracellular αAR signaling. Therefore, we hypothesized that the concentration dependent reversibility delay is explained by intracellular ligand trapping: internalized PE acting on intracellular αAR 's.

To test this hypothesis, two specialized αAR antagonists were used: prazosin, an αAR antagonist that can act at the sarcolemma and also be transported inside the cell, and CGP-12177a, a hydrophilic αAR antagonist that cannot be internalized and therefore acts only at the sarcolemma [23–26]. After recording initial images, a given concentration of prazosin or CGP-12177a with 10 $\mu\text{mol/L}$ PE was applied to the cardiac myocytes for 24 hours (Fig. 3A). Myocytes cultured with prazosin showed concentration dependent prevention of hypertrophy. Conversely, CGP-12177a did not prevent PE-induced myocyte hypertrophy. In other words, an αAR antagonist that can act inside the cell was able to prevent PE-induced hypertrophy and αAR antagonist that can only act at the sarcolemma was not. These results imply that αAR 's inside the cell are involved in PE-induced hypertrophy, which is consistent with our ligand trapping hypothesis.

To further test this mechanism, 10 $\mu\text{mol/L}$ prazosin or CGP-12177a in serum free media was administered to the myocytes after 24 hour exposure to a given concentration of PE (Fig. 3B). Myocytes treated with prazosin began decreasing in area immediately after PE washout at all concentrations of PE. This provides evidence that continued increases in cell area after extracellular PE washout was due to sustained αAR activation. The reversibility data with prazosin indicate that the largest contributor of the reversibility delay is at the level of the αAR and not due to feedback downstream of αAR 's such as from autocrine or paracrine effects [27] or nonspecificity of PE. Moreover, reversibility kinetics with prazosin compared to untreated (Figure 2A) imply that expression of GFP did not substantially affect reversal of myocyte growth.

Conversely, myocytes given CGP-12177a continued to exhibit a reversibility delay response, where hypertrophy was reversible at low (PE 10 $\mu\text{mol/L}$), but not high levels of alpha-adrenergic signaling. The final fold changes in cell area observed on day 3 with CGP-12177a were somewhat higher than what was seen in the serum-free media only condition. Based on the time course of the 0 $\mu\text{mol/L}$ PE myocytes, CGP-12177a induces minor increases in cell area on its own consistent with its mild partial agonism towards β_3 -adrenergic receptors [24] and also providing evidence that the CGP-12177a used in these experiments was active. We further demonstrate activity of CGP-12177a by demonstrating that the compound acts as a β_1 -adrenergic receptor antagonist [26] (Supplementary Fig. 7). Differences in fold change in cell area on day 1 and day 3 were tested for statistical significance using Kruskal-Wallis non-parametric one-way analysis of variance followed by a Dunn's multiple comparisons post-test test (Supplementary Table S2–3). Similarly, PE-induced increases in integrated GFP fluorescence were attenuated with prazosin and not CGP-12177a (Supplementary Fig. S8). Together these results support the hypothesis that the concentration dependent reversibility delay may be explained by intracellular ligand trapping: cellular uptake of PE with hypertrophy induced by activity at intracellular αAR 's.

3.3 Mathematical model of ligand trapping explains hypertrophy reversibility delay

To further evaluate ligand trapping's role in the reversibility of PE-induced hypertrophy, we developed an ordinary differential equation model of PE-internalization and myocyte hypertrophy (Fig. 4A). In the model, cellular uptake rate of PE is linearly related to the extracellular PE concentration. After internalization, PE can act on intracellular αAR 's and lead to increased cell area. Both PE degradation and activity at αAR 's are modeled using saturating, Michaelis-Menten form kinetics. Cell area is also influenced by constant basal growth and linear atrophy terms. Equations are provided in the Supplementary Methods.

Model parameters were estimated by nonlinear least-squares fitting to experimental data from Fig. 2A. Fig. 4B shows that the model predictions closely fit the experimental data on reversibility of PE-induced hypertrophy. As we saw experimentally, the ligand trapping model predicts the reversibility delay response, with persisting hypertrophy at high concentrations of PE. The model output shows how PE internalization allows for the time of the peak fold change in cell area to shift to later time points when myocytes are exposed to higher extracellular concentrations of PE.

The ligand trapping model makes predictions of how intracellular PE concentration and fraction of ligand-bound αAR 's (Fig. 4C) elicit sustained hypertrophic responses. After extracellular PE washout at 24 hours, internal PE concentration remains high until PE degradation reduces it back to baseline. The higher the extracellular concentration of PE myocytes were exposed to, the longer the time internal PE remains high enough to continue to act on intracellular αAR 's to increase myocyte area. The 1 mmol/L PE degradation kinetics appear distinct from the lower concentrations due to the saturating degradation of PE. Similarly, since internal levels of PE persist after washout, the fraction of receptors

bound remains high for longer in myocytes exposed to higher extracellular concentrations of PE. Intracellular α AR's, basal cell growth, and saturating PE degradation were all required to reproduce experimental observations (Supplementary Fig. S9–12). The latter may imply that an enzyme or transport mediated process is responsible for decreases in the intracellular PE over time.

A key test of this model is its ability to predict the response to prazosin (data from Fig. 3B), which was not used in model construction. Prazosin was modeled as a competitive inhibitor with K_i of 0.1 nM [28]. As seen experimentally, the model showed reversibility of PE-induced hypertrophy with addition of prazosin at all PE concentrations (Fig. 5). Experimental data for prazosin experiments had higher peak fold changes, but the reversibility kinetics for the model and experimental data are qualitatively similar. Moreover, the model is able to accurately predict longer time course data for the 1 mmol/L PE condition, which was also not used in model construction. In the 4-day time window of data collection from experiments shown in Fig. 4, the 1 mmol/L PE condition appeared irreversible, while the model structure predicted it would eventually return to baseline, only at a later time point than the lower PE concentrations. In subsequent experiments, myocytes in the 1 mmol/L PE condition began to decrease in cell area 48 hours after PE washout, with kinetics remarkably similar to the prior model predictions (Supplementary Fig. S13). These results are consistent with the ligand trapping hypothesis, that the reversibility delay in PE-induced hypertrophy is a result of internalized PE acting on intracellular α AR's.

3.4 α AR localization determined using BODIPY-prazosin is consistent with ligand trapping model

Our ligand trapping mathematical model predicts that intracellular α AR's are necessary for the concentration-dependent reversibility delay in PE-induced hypertrophy. To test this prediction we collected confocal microscopy images of myocytes with BODIPY-FL fluorescently labeled prazosin to determine the distribution of α AR's in cardiac myocytes. BODIPY-prazosin fluoresces when bound to α AR's and remains effectively non-fluorescent when unbound [29]. Images revealed that the majority of BODIPY-prazosin labeling was present inside the cardiac myocytes (Fig. 6A). Quantification of labeling from a 3D stack of images throughout the entire myocyte offer further evidence that most of the α AR's were located in the cell interior and not the sarcolemma (Fig. 6B). The distribution of BODIPY-prazosin labeling within the cells was punctate in appearance, resembling vesicles. Moreover, time-lapse images show directed movement of these punctate structures, which is consistent with the expected motion of vesicular trafficking (Supplementary Video 1). This vesicular arrangement of α AR's is consistent with previous data that showed that expressing α_{1a} AR's in R-1F cells were predominately found in intracellular organelles, including early and late endosomes [21]. These punctate vesicular structures are also apparent in BODIPY-prazosin images from adult mouse cardiac myocytes [22]. This result provides evidence of a large population of α AR's inside cardiac myocytes and is therefore consistent with the ligand trapping hypothesis.

Competitive inhibition experiments with phentolamine (an α AR antagonist) and PE were performed to quantify nonspecific binding (Fig. 6C–D). Labeling was quantified by calculating the integrated intensity of pixels above a threshold (three times the background signal) for each cell and normalizing by cell area. Phentolamine and PE both resulted in significant attenuation of the BODIPY-prazosin signal in the cardiac myocytes ($P < 0.001$). These results confirm the specificity of BODIPY-prazosin for α AR labeling and show that PE is internalized by cardiac myocytes and can bind to intracellular α AR's. CGP-12177a did not significantly attenuate the BODIPY-prazosin labeling inside the cell, providing additional evidence that CGP-12177a is not internalized by the cardiac myocytes like the α AR antagonist prazosin. These results provide evidence of intracellular α AR's and

internalization of PE by cardiac myocytes and therefore support our ligand trapping model that the reversibility delay in PE-induced hypertrophy is a result of internalized PE acting on intracellular α AR's.

4. Discussion

Automated methods that can quickly and reproducibly collect data over spatial and temporal dimensions are needed to study the complex signaling networks controlling cardiac hypertrophy. Here, we developed what is to our knowledge the first automated live-cell image acquisition and analysis approach for high-content imaging of cardiac myocyte hypertrophy. While this method has limited ability to visualize multiple proteins simultaneously or sarcomere striations compared to immunofluorescence techniques, it does not require fixation of the myocytes which allows for multiple time point imaging. Therefore, cardiac myocyte hypertrophy can be quantified and tracked in individual cells over several days. Additionally, even though plasmid-based transfection efficiency does not allow for visualization of every cell in the imaging area, it makes the cell boundaries more conspicuous, which simplifies cell boundary segmentation.

Compared to manual image collection, our method offers substantial increases in imaging speed and reproducibility, increased size of imaging area and therefore a greater number of expressing cells imaged per well/perturbation, and elimination of data collection biases in user-selected regions of cells. With our automated image analysis algorithm, we are able to quickly and robustly quantify features of cell morphology in the large image sets produced using our automated imaging system. Since the algorithm can track myocytes between subsequent images collected over multiple days, we uniquely applied this platform to study reversibility of cardiac myocyte hypertrophy.

Our approach revealed that the kinetics of PE-induced hypertrophy after PE washout is a concentration-dependent reversibility delay: hypertrophy is reversible at low and persists at high levels of α AR signaling. Data from hypertrophy reversibility experiments with specialized α AR antagonists, a mathematical model, and α AR localization images using BODIPY-prazosin support a reversibility delay mechanism of ligand trapping, where PE is internalized and acts on intracellular α AR's. In addition to our BODIPY-prazosin data in neonatal cardiac myocytes, recent evidence has shown a large population of α_1 AR's in adult cardiac myocytes [22]. Moreover, expressing α_{1a} AR's in R-1F cells were predominately found in intracellular organelles [21] and 40% of native α_1 AR's in human smooth muscle cells were intracellular [30]. Prazosin, an α AR antagonist that can act at the sarcolemma and also be transported inside the cell, reversed PE-induced hypertrophy. In contrast, CGP-12177a, a membrane impermeable α AR antagonist, did not reverse PE-induced hypertrophy. The prazosin and CGP-12177a results indicate that the reversibility delay mechanism is primarily at the receptor level and not predominately the result of downstream positive feedback. Additionally, only a mathematical model incorporating intracellular α AR's could reproduce the experimentally observed reversibility delay. Evidence of intracellular α AR's was obtained using BODIPY fluorescently labeled prazosin and competitive binding experiments with PE provide evidence for internalization of PE and prazosin but not CGP-12177a. A similar ligand trapping mechanism may be important in other signaling pathways in the cardiac hypertrophy signaling network. Functional intracellular receptors have been identified for angiotensin II [31], endothelin-A and B [32], and β -adrenergic receptors [33] in cardiac myocytes.

α_1A and α_1B receptor subtypes have been shown to colocalize with G α_q and PLC β_1 at the nuclear membrane in adult mouse cardiac myocytes [22]. Moreover, membrane permeable α_1 AR antagonist prazosin blocked phosphorylation of ERK in adult mouse cardiac

myocytes while membrane impermeable $\alpha 1$ AR antagonist CGP-12177a did not [22]. To better characterize this mode of signaling, additional studies demonstrating interaction of G α q with intracellular α AR's such as with FRET microscopy and experiments showing location of G α q activity would be valuable. Additionally, experiments characterizing the trafficking of vesicular α AR's and downstream signaling proteins warrant further study.

Hypertrophy reversibility delay and ligand trapping results may have implications for disease progression and treatment for heart failure. Endogenous α AR agonists norepinephrine and epinephrine are released in response to cardiac stress. The reversibility delay result implies that through ligand trapping, hypertrophy signaling can continue for a significant time after transient exposure to circulating catecholamines. Catecholamine uptake data show that cardiac myocytes can internalize substantial levels of norepinephrine within 5 minutes [22]. This memory mechanism, while potentially providing cardioprotection for future insults, could also accelerate the development of hypertrophy and heart failure. Moreover, α AR agonist internalization would complicate therapeutic approaches aimed at blocking this pathway. Through internalization, the cell could circumvent antagonists that act only at the sarcolemma, as seen here with myocytes treated with CGP-12177a. Therefore development of pharmacologic agents that can be readily internalized by the cell or prevent uptake of hypertrophic agonists may be important for successful treatment of cardiac hypertrophy.

Here we used cultured neonatal rat ventricular myocytes in our imaging platform to study cardiac myocyte hypertrophy reversibility. While cultured cells do not fully replicate the complex 3D environment of the heart, they are necessary for the scalable high-throughput experiments needed for systems-wide analysis of hypertrophy signaling networks. Previous studies have demonstrated reversibility in patients after surgical interventions [7–9] and in mice with drug treatments [4–6]. Moreover it was recently shown that hypertrophy and systolic dysfunction induced by constitutively active calcineurin was reversible by solely turning off calcineurin activity, without the requiring any additional treatment [34]. This study has interesting implications concerning the reversibility of hypertrophy when the underlying cause is removed. Furthermore, the reversibility of signaling downstream of calcineurin is consistent with our data that PE-induced hypertrophy is reversible. Reversibility of other hypertrophic pathways and specific circumstances that may disrupt this reversibility requires further study.

Comprehensive studies in cultured cells are needed to better understand signaling network organization, cross-talk, and mechanisms of hypertrophy reversibility. This knowledge will help improve pharmacologic and genetic target selection for treatment of cardiac hypertrophy. Moreover, the high-throughput image acquisition and analysis techniques shown here could be extended to be used in high-throughput drug or RNAi screens or with human stem-cell derived cardiac myocytes [35].

In summary, we developed an automated image acquisition and analysis approach for quantifying changes in morphology of individual cardiac myocytes over time. This methodology can accommodate the large number of pharmacologic and genetic perturbations needed to better study the biological circuits controlling cardiac hypertrophy. This approach revealed that PE-induced hypertrophy exhibits a concentration-dependent reversibility delay that can be explained by intracellular ligand trapping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Lindsay McClellan and Renata Polanowska-Grabow for technical assistance. This work was supported by the National Institutes of Health (grant HL094476 to J.S., training grant HL007284 supporting K.R.) and a National Science Foundation Graduate Fellowship (to K.R.).

Abbreviations List

αAR	α-adrenergic receptor
PE	phenylephrine

References

- Hill JA, Olson EN. Cardiac plasticity. *N Engl J Med*. 2008; 358(13):1370–80. [PubMed: 18367740]
- Mudd JO, Kass DA. Tackling heart failure in the twenty-first century. *Nature*. 2008; 451(7181): 919–28. [PubMed: 18288181]
- Koitaishi N, Kass DA. Reverse remodeling in heart failure-mechanisms and therapeutic opportunities. *Nat Rev Cardiol*. 2011;1–11.
- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, et al. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med*. 2005; 11(2):214–22. [PubMed: 15665834]
- Xu D, Li N, He Y, Timofeyev V, Lu L, Tsai H-J, et al. Prevention and reversal of cardiac hypertrophy by soluble epoxide hydrolase inhibitors. *Proc Natl Acad Sci USA*. 2006; 103(49): 18733–8. [PubMed: 17130447]
- Moens AL, Takimoto E, Tocchetti CG, Chakir K, Bedja D, Cormaci G, et al. Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. *Circulation*. 2008; 117(20):2626–36. [PubMed: 18474817]
- Zafeiridis A, Jeevanandam V, Houser SR, Margulies KB. Regression of cellular hypertrophy after left ventricular assist device support. *Circulation*. 1998; 98(7):656–62. [PubMed: 9715858]
- Kuhn M, Voss M, Mitko D, Stypmann J, Schmid C, Kawaguchi N, et al. Left ventricular assist device support reverses altered cardiac expression and function of natriuretic peptides and receptors in end-stage heart failure. *Cardiovasc Res*. 2004; 64(2):308–14. [PubMed: 15485690]
- Birks EJ, George RS, Hedger M, Bahrami T, Wilton P, Bowles CT, et al. Reversal of severe heart failure with a continuous-flow left ventricular assist device and pharmacological therapy: a prospective study. *Circulation*. 2011; 123(4):381–90. [PubMed: 21242487]
- Lusis AJ, Weiss JN. Cardiovascular networks: systems-based approaches to cardiovascular disease. *Circulation*. 2010; 121(1):157–70. [PubMed: 20048233]
- Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006; 7(8):589–600. [PubMed: 16936699]
- Evans JG, Matsudaira P. Linking microscopy and high content screening in large-scale biomedical research. *Methods Mol Biol*. 2007; 356:33–38. [PubMed: 16988393]
- Bass GT, Ryall KA, Katikapalli A, Taylor BE, Dang ST, Acton ST, et al. Automated image analysis identifies signaling pathways regulating distinct signatures of cardiac myocyte hypertrophy. *J Mol Cell Cardiol*. 2012;10.1016/j.yjmcc.2011.11.009
- Jentzsch C, Leierseder S, Loyer X, Flohrschütz I, Sassi Y, Hartmann D, et al. A phenotypic screen to identify hypertrophy-modulating microRNAs in primary cardiomyocytes. *J Mol Cell Cardiol*. 2012; 52(1):13–20. [PubMed: 21801730]
- Prasad KMR, Xu Y, Yang Z, Acton ST, French BA. Robust cardiomyocyte-specific gene expression following systemic injection of AAV: in vivo gene delivery follows a Poisson distribution. *Gene Ther*. 2011; 18:43–52. [PubMed: 20703310]
- Otsu N. A threshold selection method from gray-level histograms. *IEEE Trans Syst Man and Cybern*. 1979; 9(1):62–6.

17. Vincent L, Soille P. Watersheds in digital spaces: an efficient algorithm based on immersion simulations. *IEEE Trans Pattern Anal Mach Intell.* 1991; 13(6):583–97.
18. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 2006; 7(10):R100. [PubMed: 17076895]
19. Zia Y, Rajapurohitam V, Cook MA, Karmazyn M. Inhibition of phenylephrine induced hypertrophy in rat neonatal cardiomyocytes by the mitochondrial K_{ATP} channel opener diazoxide. *J Mol Cell Cardiol.* 2004; 37:1063–67. [PubMed: 15522283]
20. Barron AJ, Finn SG, Fuller SJ. Chronic activation of extracellular-signal-regulated protein kinases by phenylephrine is required to elicit a hypertrophic response in cardiac myocytes. *Biochem J.* 2003; 371:71–79. [PubMed: 12513686]
21. Pediani JD, Colston JF, Caldwell D, Milligan G, Daly CJ, Mcgrath JC. β -arrestin-dependent spontaneous α 1a-adrenoceptor endocytosis causes intracellular transportation of α -blockers via recycling compartments. *Mol Pharmacol.* 2005; 67(4):992–1004. [PubMed: 15626751]
22. Wright CD, Chen Q, Baye NL, Huang Y, Healy CL, Kasinathan S, et al. Nuclear α 1-adrenergic receptors signal activated ERK localization to caveolae in adult cardiac myocytes. *Circ Res.* 2008; 103(9):992–1000. [PubMed: 18802028]
23. Levin MC, Marullo S, Muntaner O, Andersson B, Magnusson Y. The myocardium-protective Gly-49 variant of the β 1-adrenergic receptor exhibits constitutive activity and increased desensitization and down-regulation. *J Biol Chem.* 2002; 277(34):30429–35. [PubMed: 12034720]
24. Staehelin M, Simons P, Jaeggi K, Wigger N. CGP 12177: A hydrophilic β -adrenergic receptor radioligand reveals high affinity binding of agonists to intact cells. *J of Biol Chem.* 1983; 258(6):3496–3502. [PubMed: 6131886]
25. Brahmadevara N, Shaw AM, MacDonald A. α 1-adrenoceptor antagonist properties of CGP 12177A and other β -adrenoceptor ligands: evidence against β 3- or atypical β -adrenoceptors in rat aorta. *Br J Pharmacol.* 2004; 142(4):781–7. [PubMed: 15205310]
26. Brahmadevara N, Shaw AM, MacDonald A. Evidence against β 3-adrenoceptors or low affinity state of β 1-adrenoceptors mediating relaxation in rat isolated aorta. *Br J Pharmacol.* 2003; 138(1):99–106. [PubMed: 12522078]
27. Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol.* 1997; 59(56):551–71. [PubMed: 9074777]
28. Nishimura J, Kanaide H, Nakamura M. Binding of [^3H]Prazosin to porcine aortic membranes: interaction of calcium antagonists with alpha-1 adrenoceptors. *J Pharmacol Expl Ther.* 1986; 236(3):789–93.
29. Daly CJ, Milligan CM, Milligan G, Mackenzie JF, Mcgrath JC. Cellular localization and pharmacological characterization of functioning alpha-1 adrenoceptors by fluorescent ligand binding and image analysis reveals identical binding properties of clustered and diffuse populations of receptors. *J Pharmacol Expl Ther.* 1998; 286(2):984–90.
30. Mackenzie JF, Daly CJ, Pediani JD, McGrath JC. Quantitative imaging in live human cells reveals intracellular α 1-adrenoceptor ligand-binding sites. *J Pharmacol Expl Ther.* 2000; 294(2):434–43.
31. Tadevosyan A, Maguy A, Villeneuve LR, Babin J, Bonnefoy A, Allen BG, et al. Nuclear-delimited angiotensin receptor-mediated signaling regulates cardiomyocyte gene expression. *J of Biol Chem.* 2010; 285(29):22338–22349. [PubMed: 20463030]
32. Boivin B, Chevalier D, Villeneuve LR, Rousseau E, Allen BG. Functional endothelin receptors are present on nuclei in cardiac ventricular myocytes. *J of Biol Chem.* 2003; 278(31):29153–63. [PubMed: 12756260]
33. Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, et al. Functional β -adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. *Cardiovasc Res.* 2006; 71(1):69–78. [PubMed: 16631628]
34. Berry JM, Le V, Rotter D, Battiprolu PK, Grinsfelder B, Tannous P, et al. Reversibility of adverse, calcineurin-dependent cardiac remodeling. *Circ Res.* 2011; 109:407–17. [PubMed: 21700928]
35. Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flügel L, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med.* 2010; 363(15):1397–409. [PubMed: 20660394]

Highlights

- Automated image acquisition and analysis approach for cardiac myocyte hypertrophy
- Tracks changes in measurements of individual myocytes over time
- Reversibility of hypertrophy shown in a scalable cell culture model
- Hypertrophy reverses at low and persists at high levels of α -adrenergic signaling
- Delay in reversal explained by a ligand trapping model.

Figure 1. High-throughput image acquisition and analysis platform can track morphological dynamics of individual cardiac myocytes

A) Scripts to control the microscope's automated stage allowed for highly reproducible imaging of 5×5 mosaics images of GFP-labeled cardiac myocytes within each well of a 96-well plate. These mosaic images maximize the cell data collected per well/perturbation, including approximately 100–200 cells per well. **B)** Example output cell boundary segmentations from automated image analysis algorithm. Using these segmentations, a variety of cardiac myocyte morphology measurements can be calculated. **C)** Example output from cardiac myocyte tracking over 3 days. Cells are labeled and tracked between measurements based on the proximity to the cell's location on the preceding day.

Figure 2. Hypertrophy is reversible at low and persists at high levels of α -adrenergic signaling
A) Time course data of median (~400 cells per condition) fold change in cell area of cardiac myocytes after 24 hours exposure to a given concentration of PE in serum free media. On day 1 the agonist was washed out and replaced with serum free media. Error bars are $\pm$ SE. **B)** Representative images with labeled cell areas (Scale bar: 10 μ m) of segmented cardiac myocytes exposed to 1 mmol/L PE (top) and 1 μ mol/L PE (bottom). Cells exposed to 100 μ mol/L PE continue to increase in size after the agonist is washed out of the extracellular media.

Figure 3. Hypertrophy is reversed by membrane permeable α AR-antagonist but not membrane impermeable antagonist

A) Median (~350 cells per condition) fold change in cell area of cardiac myocytes exposed to 10 μ mol/L of PE with a given concentration of prazosin (left) or CGP-12177a (right) for 24 hours. prazosin and CGP-12177a are both alpha adrenergic receptor antagonists. Prazosin can act at the sarcolemma and also be transported inside the cell and CGP-12177a can only act at the sarcolemma. Error bars are $\pm$ SE. **B)** Time course data of median (~1000 cells per condition) fold change in cell area of cardiac myocytes after 24 hours exposure to a given concentration of PE. On day 1, PE was washed out and cells were cultured in 10 μ mol/L prazosin (left) or 10 μ mol/L CGP-12177a (right) in serum free media. Error bars are $\pm$ SE. Prazosin reverses PE-induced hypertrophy. CGP-12177a does not affect hypertrophy reversibility, supporting the hypothesis that internalized PE acting on nuclear α AR's may explain the reversibility delay.

Figure 4. Concentration-dependent reversibility delay may be explained by a ligand trapping model

A) Schematic of the mathematical model. PE is internalized by the cell and acts on intracellular α AR's, which results in increased cell size. **B)** Results of nonlinear least squares fit of the model to experimental data (points). **C)** Model-predicted time courses for internal PE concentration (left) and fraction of α AR's with bound PE (right). At high concentrations, internal PE concentration and fraction of receptors bound remains high after agonist is washed out of the extracellular media, allowing for further increases in myocyte hypertrophy.

Figure 5. Ligand trapping model predicts reversibility of hypertrophy with prazosin and longer time course data of 1 mmol/L PE treated myocytes

A) Mathematical model predictions and experimental data (points) of fold change in cell size of myocytes exposed to PE and then prazosin after agonist washout. As seen experimentally, the model predicts that hypertrophy is reversed by membrane permeable α AR antagonist, prazosin ($K_i=0.1$ nM), after 24 hour exposure to a given concentration of PE.

Figure 6. Intracellular α AR's in cardiac myocytes imaged using fluorescently labeled BODIPY-prazosin

A) Cardiac myocytes in a 10 nmol/L solution of BODIPY-prazosin were imaged using a confocal laser scanning microscope and a 63X oil objective. The distribution of α AR's in the myocytes is predominately intracellular, as predicted by the ligand trapping model. The α AR labeling occurred in punctate vesicles spread throughout the cytoplasm. **B)** 3D Distribution of BODIPY-prazosin labeling indicates large population of intracellular α AR's. Integrated intensity of above threshold signal in the BODIPY-prazosin channel normalized by cell area was computed at 0.5- μ m intervals throughout cardiac myocytes. Results for a representative cell are shown above. Labeling is highest in the center indicating that the majority of α AR's are intracellular, which is consistent with the ligand trapping model. **C)** Median and interquartile range of integrated intensity of above background BODIPY-prazosin signal normalized by cell area for competitive binding experiments of 10 nmol/L BODIPY-prazosin with 10 μ mol/L phentolamine, 1 mmol/L PE, or 100 μ mol/L CGP-12177a. N~80 cells per condition. Results with PE and phentolamine show high specificity of BODIPY-prazosin in labeling α AR's and results with CGP-12177a provide further evidence that CGP-12177a is not internalized like the α AR antagonist prazosin. ***P<0.0001 **D)** Representative images with segmented cell boundaries from the BODIPY-prazosin competitive binding experiments for each condition. The quantification of the labeling (integrated intensity/cell area) for the pictured cell is indicated below.