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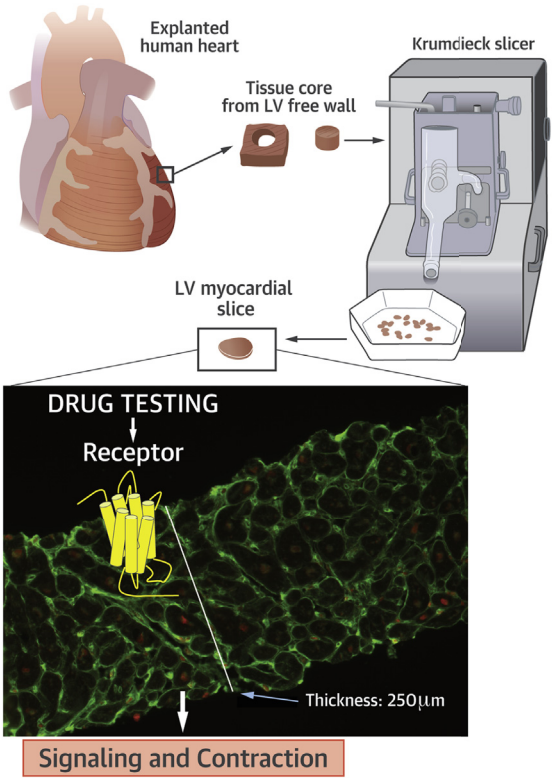
NOVEL TRANSLATIONAL METHODS

A Myocardial Slice Culture Model Reveals Alpha-1A-Adrenergic Receptor Signaling in the Human Heart

R. Croft Thomas Jr, MD,[a,b] Abhishek Singh, MD, PhD,[a,b] Patrick M. Cowley, PhD,[a,b] Bat-Erdene Myagmar, MD, PhD,[a,b] Megan D. Montgomery, PhD,[a,b] Philip M. Swigart, BS,[b] Teresa De Marco, MD,[b] Anthony J. Baker, PhD,[a,b] Paul C. Simpson, MD[a,b]

VISUAL ABSTRACT



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HIGHLIGHTS

- **Model for translational studies**
- **Human LV slices**
- **Simple, high throughput, viable**
- **Assays signaling and contraction**
- **Supports viral transduction**
- **Useful for proof-of-concept in man**
- **Shows the α1A-AR functions in human heart**

From the [a]Department of Medicine, VA Medical Center, San Francisco, California; and the [b]Department of Medicine, University of California, San Francisco, San Francisco, California. Dr. Singh's current address is the Department of Medicine, Temple University, Philadelphia, Pennsylvania. Support was from the National Institutes of Health (NIH) (grant HL31113 to Dr. Simpson), the Department of Veterans Affairs (Drs. Baker and Simpson), the Western States Affiliate of the American Heart Association (Drs. Thomas, Myagmar, and Baker), the PhRMA Foundation (Dr. Montgomery), and an NIH training grant from the University of California, San Francisco (UCSF) Cardiovascular Research Institute (grant 5T32HL007731 to Dr. Singh). The Northern California Institute for Research and Education administered grants, and the Veterans Affairs Medical Center, San Francisco, California, provided resources. UCSF owns a patent with Dr. Simpson as inventor for use of A61603 in heart failure, and Dr. Simpson and Montgomery are involved in a company to pursue this. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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SUMMARY

The authors used 52 nonfailing and failing human hearts to develop a simple, high throughput left ventricular myocardial slice model that is stable by ATP and viability assays for at least 3 days. The model supports studies of signaling, contraction, and viral transduction. They use the model to show for the first time that the alpha-1A-adrenergic receptor, which is present at very low abundance in the human myocardium, activates cardioprotective ERK with nanomolar EC50 in failing heart slices and stimulates a positive inotropic effect. This model should be useful for translational studies, to test whether molecules discovered in basic experiments are functional in the human heart. (J Am Coll Cardiol Basic Trans Science 2016;1:155–67) Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Basic and pre-clinical research has identified numerous signaling molecules that might be efficacious targets for drugs to treat heart failure and myocardial disease (1). Translation of these findings to clinical use could be facilitated if the activity of these signaling molecules in human ventricular myocytes was known. Isolated or cultured human ventricular myocytes are useful for assays of contraction and calcium handling (2–4). However, surprisingly few studies use myocytes for biochemical signaling assays, such as immunoblot (5,6). One challenge with human ventricular myocytes is the technical difficulty of isolation and the relatively low cell yields, making it difficult to do biochemical assays with numerous replicates.

Isolated right ventricular trabeculae can assay protein phosphorylation (7), but availability of trabeculae with the requisite small size can be limiting (5). Chopped or hand-cut myocardial pieces can also serve to explore signaling (8,9), but limited viability, technical difficulty, or applicability to other assays, such as contraction, are limitations.

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As an alternative approach to a human biochemical signaling model, we studied thin slices made from cores of left ventricular (LV) myocardium. Thin slices have a long history of use in other tissues, but there is minimal precedent in heart, especially human (10). Others describe human myocardial slices, but there is no report of biochemical signaling, likely due in part at least to limited stability (11) or low numbers of samples (12,13).

Here, we describe a simple, reproducible, high-throughput method to generate large numbers of slices from cores of human LV myocardium. We show that these slices are stable and viable over several days, ample time to assay signaling, contraction, and viral transduction. As a test case, we study the effects of the highly selective α1A-adrenergic receptor (AR) agonist A61603. The α1A-AR is expressed in human myocardium, but levels are very low, only 3% of total adrenergic receptors (ARs) in the nonfailing human LV, and 7% in the failing LV (14). Whether this low level of α1A-ARs mediates detectable signaling or function in the human myocardium is unknown. The slice model shows for the first time that human LV α1A-ARs couple to ERK activation with high potency, and also stimulate a positive inotropic response.

ABBREVIATIONS AND ACRONYMS

Ab = antibody
AR = adrenergic receptor
ATP = adenosine triphosphate
BDM = 2,3-butanedione monoxime
β-MyHC = beta-myosin heavy chain
EC50 = half-maximal effective concentration
GFP = green fluorescent protein
ISO = isoproterenol
LV = left ventricle/ventricular
LVAD = left ventricular assist device
OCT = optical cutting temperature compound
PBS = phosphate-buffered saline
PE = phenylephrine
pERK = phospho-ERK
PKD1 = protein kinase D1
pPLN = phosphorylated phospholamban
UCSF = University of California, San Francisco

METHODS

PATIENTS. With the approval of the University of California, San Francisco (UCSF), Committee for Human Research, we obtained LV free wall tissue from hearts removed at the time of transplant at UCSF, or from organ donors whose hearts were not transplanted for technical reasons. Full informed consent was obtained from all UCSF transplant recipients before surgery. The Donor Network West (formerly California Transplant Donor Network) provided the unused donor hearts and obtained informed consent from the donor's next of kin. Hearts were collected and transported to the laboratory in cold cardioplegia.

MAJOR EQUIPMENT. We used the following major equipment for slice preparation and culture, all from Alabama Research and Development (Munford, Alabama): coring press (MD5000/53000); cylindrical coring tool 8-mm diameter (MP0144); tissue embedding unit (MD2299); Krumdieck tissue slicer (MD4000); titanium meshes (MD0036); and slice incubation unit (MD2500).

HEART COLLECTION. Hearts were explanted, and the coronary arteries were immediately perfused via the aorta with 1 l of ice-cold cardioplegia solution. Complete blood washout was visualized. Hearts were transported to the laboratory in ice-cold cardioplegia, typically within 2 h after explant.

Cardioplegia for heart collection (Plegisol, 0409-7969-05, Hospira, Lake Forest, Illinois): NaCl 110 mmol/l; KCl 16 mmol/l; $CaCl_2$-$2H_2O$ 1.2 mmol/l; $NaHCO_3$ 10 mmol/l; $MgSO_4$-$6H_2O$ 16 mmol/l; that is ice-cold (pH 7.8) and sterile.

CORING. Working on an open bench in ice-cold cardioplegia, 2-cm by 2-cm sections were cut from the middle of the LV free wall, and 8-mm diameter cores were generated using a coring press with an 8-mm cylindrical coring tool. Areas with obvious LV scar were avoided. Care was taken to cut the cores perpendicular to the myofiber long axis. After coring, a razor blade was used to remove fat and trabeculae from the epicardial and endocardial surfaces. Cores were embedded in 2% low melting temperature agarose using the tissue embedding unit.

Agarose embedding buffer for cores: slicing buffer (in the following text); low melting agarose 20 mg/ml (Agarose II, 0815, Amresco, Solon, Ohio); sterile, stored at 40°C, and poured over the core in ice-cold tissue embedding unit.

SLICING. The embedded core was oriented in the Krumdieck tissue slicer with the endocardial surface toward the blade, such that the cutting plane was parallel to the myocyte long axis; a supplied weight maintained downward pressure. Slice thickness was set at 250 μm. The instrument passed the core repeatedly and automatically across a replaceable stainless steel blade while immersed in 4°C, sterile buffer. Circulating buffer floated the resultant slices into a glass trap and a collecting tray.

Modified calcium-free Tyrode's for heart slicing: NaCl 111 mmol/l; KCl 16 mmol/l; HEPES 10 mmol/l; $NaHCO_3$ 4.2 mmol/l; $MgSO_4$-$7H_2O$ 0.4 mmol/l; glucose 5.6 mmol/l; 2,3-butanedione monoxime (BDM) (B0753, Sigma-Aldrich, St. Louis, Missouri) 20 mmol/l; (pH 7.2) at 4°C in air. Components are added to sterile water and filter-sterilized.

CALCIUM REINTRODUCTION. Slices were transferred using a cut-off pipette into a 50-ml tube with ice-cold calcium reintroduction buffer. After 10 min, additional $CaCl_2$ was added at 10-min intervals to bring the [Ca] successively to 25, 50, 100, 200, 400, 700, and 1,000 μmol/l.

Calcium reintroduction buffer: Modified calcium-free Tyrode's as in the preceding text. Additionally: blebbistatin (ab120425, Abcam, Cambridge, Massachusetts) 0.1 mmol/l; BDM 40 mmol/l; adenosine triphosphate (ATP) 2 mmol/l; ice-cold, sterile (pH 7.2), and in air.

SLICE CULTURE. Using wide forceps to lift the slices from underneath without damage (Millipore filter forceps #XX6200006P, EMD Millipore, Billerica, Massachusetts), 2 to 4 slices were placed onto a titanium mesh in each well of a 6-well culture tray with 2 ml of culture medium. Four trays were placed on a dedicated slice incubation unit in a standard culture incubator at 37°C with 2% CO_2. The slice incubation unit rotated slices through air alternating with medium at 1 rpm. Slices were harvested at intervals for assays, without a change in medium.

Culture medium: MEM Eagle with Hanks' Balanced Salt Solution; HEPES 10 mmol/l; ATP 2 mmol/l; penicillin 50 U/ml; vitamin B_{12} 1.5 mmol/l; bovine calf serum 10% (HyClone SH30073.03, GE Healthcare Life Sciences, Logan, Utah); blebbistatin (Abcam ab120425) 0.1 mmol/l. Note: BDM (B0753, Sigma-Aldrich) 50 mmol/l was substituted for blebbistatin, only with slices destined for contraction studies, because blebbistatin has slow washout.

DOXORUBICIN DIFFUSION. Following slice generation, slices were incubated in culture medium with doxorubicin HCl 100 μmol/l (#2252, Tocris Bioscience, Minneapolis, Minnesota). After varying times (1 min or longer), slices were rinsed in ice-cold phosphate-buffered saline (PBS) for 10 min, then incubated at 4°C successively in 4% formaldehyde overnight, 15% sucrose in PBS for 2 h, then 30% sucrose in PBS overnight. Slices were embedded in optical cutting temperature compound (OCT) (#27050, Ted Pella, Redding, California), and 6-μm cross sections were cut with a cryostat. Sections were stained with wheat germ agglutinin 5 μg/ml for 15 min, washed in PBS for 10 min, air dried, and mounted with Fluoromount-G (#0100-01, SouthernBiotech, Birmingham, Alabama). Images were captured by fluorescent microscopy using a 20× objective, excitation 540 to 525 nm, emission 605 to 655 nm.

BETA-MYOSIN HEAVY CHAIN AND PHOSPHO-ERK1/2 IMMUNOHISTOCHEMISTRY. Slices were fixed using 4% formaldehyde and incubated at 4°C in 4% sucrose in PBS for 1 h, 15% sucrose in PBS for 4 h, and 30% sucrose in PBS overnight. Slices were sunk into OCT and snap-frozen in liquid nitrogen. Cryosections were cut parallel to the long axis of the slice, resulting in tangential sections, placed onto glass slides, washed in PBS for 10 min, and then incubated in PBS with 1% triton X100 for 30 min at room temperature. Excess liquid was removed, and a barrier pen was used to trace a border around the sections.

Sections were placed in a humidified chamber, blocked with 125 μl of 5% goat serum in PBS, and rocked for 20 min at room temperature. Excess fluid was suctioned off, and 125 μl of staining buffer with primary antibody (Ab) was added (PBS with 0.2% Triton X-100 and 1% goat serum with Ab to

beta-myosin heavy chain [β-MyHC] or Ab to phospho-ERK [pERK]). Sections were incubated overnight in a humidified chamber at 4°C, washed 3 times with 125 μl of TBS-T for 2 min each, and washed 2 times with PBS for 5 min. Sections were then incubated with 125 μl of staining buffer with secondary Ab, rocked at room temperature for 1 h in a humidified chamber, washed 4 times with 125 μl of TBS-T for 2 min each wash, and finally washed with PBS 2 times for 5 min each wash. Excess fluid was removed and sections were mounted with Fluoromount-G.

The β-MyHC Ab was a mouse monoclonal clone NOQ7.5.4D (#M8421, 1:200, Sigma-Aldrich). The pERK Ab was a rabbit monoclonal against ERK1 phosphorylated at threonine 202 and tyrosine 204, and ERK2 phosphorylated at threonine 185 and tyrosine 187 (D13.14.4E, XP, #4370; 1:50, Cell Signaling Technology, Danvers, Massachusetts). The secondary Ab for β-MyHC was goat anti-mouse IgG conjugated to Cy3 (#115-166-003, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania), and the secondary Ab for pERK was goat anti-rabbit IgG conjugated to Alexa 488 (#A11034, Life Technologies-Molecular Probes, Carlsbad, California), both at 1:400.

ATP AND MTT ASSAYS FOR VIABILITY. Quantifying ATP content and mitochondrial dehydrogenase activity (MTT assay) assessed slice viability.

To measure ATP, slices were taken immediately after slicing, or were cultured for varying times, washed thoroughly in PBS, frozen in liquid nitrogen in 1.5-ml Eppendorf tubes, and stored at −80°C. Liquid nitrogen was added to the tube, and slices were pulverized to a fine powder with a pestle, not allowing thawing. Immediately, 1 ml of ice-cold 10% trichloroacetic acid was added. Samples were allowed to thaw on ice, then vortexed, and precipitated protein was pelleted at 13,226 × *g* for 10 min. The ATP-containing supernatant was separated from the pellet and neutralized with a 1:100 dilution of HEPES 10 mmol/l. ATP was quantified by luciferase assay (CellTiter-Glo Luminescent Cell Viability Assay, #G7572, Promega, Madison, Wisconsin), detected by the GloMax-Multi + Detection System (#9311-010, Promega) against ATP standards 1 nmol/l to 1 μmol/l. The protein pellet was solubilized in SDS 1% and HEPES 0.1 mol/l, sonicated for 5 s, left at room temperature overnight, and quantified by the Lowry method against BSA. ATP was referenced to slice protein.

To determine mitochondrial dehydrogenase activity, MTT reagent (MTT Cell Proliferation Assay, #30-1010K, ATCC, Manassas, Virginia) 200 μl was added to slices in 2 ml of culture medium for 30 min at 37°C. The slices were rinsed in PBS, and frozen at −80°C. The purple formazan precipitate was solubilized in 1 ml of DMSO (#D5879, Sigma-Aldrich) for 1 h at 37°C with vortexing once at 30 min, and absorbance in 100 μl was quantified relative to DMSO alone, on the GLOMAX Multi+ Detection System. Slices were dabbed dry with Kimwipes, and weighed. Absorbance was referenced to slice wet weight, which was highly correlated with slice protein by Bradford ($r^2 = 0.9$, $p < 0.0001$, $n = 10$).

IMMUNOBLOT FOR PHOSPHORYLATED PHOSPHOLAMBAN AND pERK. Slices in culture medium for 1 day were incubated with L-isoproterenol HCl (ISO) (#I6504, Sigma-Aldrich), DL-propranolol HCl (#318-98-9, Sigma-Aldrich), or A61603 (#1052, Tocris Bioscience). After varying times, slices were rinsed, frozen in liquid nitrogen in 1.5-ml Eppendorf tubes, and stored at −80°C. Liquid nitrogen was added to the tube, and slices were pulverized to a fine powder with a pestle, not allowing thawing, sonicated 5 s in 0.75 ml of 1% SDS with HEPES 100 mmol/l, phosphoSTOP (#04 906 837 001, Roche Applied Science, Indianapolis, Indiana), and protease inhibitor (complete Mini, #11836 153 001, Roche Applied Science), vortexed, and centrifuged at 13,226 ×*g* for 5 min at room temperature. Protein in the supernatant was quantified by the Lowry method, and 10 to 20 μg of protein per lane was used in immunoblot for phosphorylated phospholamban (pPLN) and pERK. Protein loading was confirmed by Coomassie blue staining of the blot.

The Ab for PLN phosphorylated at serine 16 was an Upstate rabbit polyclonal Ab #07-052 (1:10,000, EMD Millipore). The Ab for ERK1 phosphorylated at threonine 202 and tyrosine 204, and ERK2 phosphorylated at threonine 185 and tyrosine 187 was a Cell Signaling Technology rabbit monoclonal Ab #4370 (1:1,000). The Ab for total ERK1/2 was a Cell Signaling rabbit polycloncal Ab #9102, and the Ab for total PLN was an Upstate mouse monoclonal clone A1 #05-205. The secondary Ab was horseradish peroxidase-linked goat anti-rabbit IgG (#7074, 1:10,000, Cell Signaling Technology) or horse anti-mouse IgG (#7076, 1:10,000 Cell Signaling Technology).

SLICE CONTRACTION. Linear strips approximately 300 to 400 μm wide were dissected from myocardial slices in culture medium, then placed in a muscle chamber (3 × 3 × 15 mm) and mounted on stainless steel pins. One end of the strip was mounted to a force transducer (AE-801, Kronex, Oakland, California), and the other end to a micromanipulator. Strips were superfused at 5 ml/min for 1 h at room temperature in Krebs-Henseleit solution (in mmol/l: NaCl 112, KCl 5, $MgCl_2$ 1.2, glucose 10, $NaHCO_3$ 24,

Na_2SO_4 1.2, NaH_2PO_4 2.0, $CaCl_2$ 0.2), oxygenated with 95% O_2, 5% CO_2. The calcium level of the solution was gradually increased to 1.8 mmol/l, and the temperature was increased to 37°C. The strip was stimulated to contract at 0.2 Hz using platinum wire electrodes at maximal voltage. Optimal length of the strip was determined by adjusting the length to achieve the highest force production. Strips were stimulated with AR agonists as described in the Results section, using A61603, phenylephrine (PE) (#P-6126, Sigma-Aldrich), or ISO.

SLICE TRANSDUCTION WITH ADENOVIRUS. Slices were incubated in culture medium on day 0 with pAdEasy-1 (Stratagene, Agilent Technologies, Santa Clara, California) with the CMV promoter driving human protein kinase D1 (PKD1) with an N-terminal green fluorescent protein (GFP) tag. After 24 h culture at 37°C, slices were fixed by shaking in 4% formaldehyde for 10 min at 4°C and transferred to 4°C PBS for 72 h. Slices were treated with increasing concentrations of sucrose in PBS, embedded in OCT, and cryosectioned at 6 μm with a cross-sectional orientation.

Sections at room temperature were rinsed in PBS for 10 min, permeabilized in 0.25% Triton PBS for 10 min, blocked with 5% normal goat serum in PBS for 1 h, rinsed in PBS twice for 10 min per rinse, incubated 1 h with Alexa 488-conjugated rabbit polyclonal Ab to GFP (#A21311, Molecular Probes) diluted 1:400 in PBS, rinsed in PBS twice for 10 min per rinse, and mounted with Fluoromount-G. Fluorescent photomicrographs were taken with an epifluorescent microscope using the green channel.

STATISTICS. Results are mean ± SE. Dose-response curves were fit and significant differences ($p < 0.05$) were tested in GraphPad Prism v5.0d (GraphPad Software, La Jolla, California). Regular 2-way analysis of variance was used for Figure 3, and 95% confidence limits were calculated for Figures 4 to 6. We used the D'Agostino and Pearson omnibus test for normality.

RESULTS

DEVELOPMENT OF A HIGH-THROUGHPUT SLICE MODEL FOR SIGNALING. We developed and validated the slice model over a period of 5 years, using 18 transplant recipient hearts (designated failing), and 34 unused donor hearts (nonfailing). For failing hearts, average patient age was 52 years (range 27 to 72 years), with 61% male. For nonfailing, the average age was 56 years (range 19 to 72 years), with 65% male. Most transplant patients were receiving beta-blockers (22%), were treated with an LV assist device (LVAD) (50%), or both (11%).

Figure 1 illustrates slice generation and culture. Each step of the procedure was modified and tested during development, and a detailed final protocol is given in the Methods section. The Krumdieck instrument cut LV cores into slices 250 μm thick at a rate of ~10 to 20 per min, and the slices floated into a collection tray.

Calcium was gradually reintroduced to 1 mmol/l, and 2 to 4 slices were placed on a titanium mesh in each well of a 6-well culture tray with 2 ml of medium. Four or more trays were placed on an inclined incubation unit in a CO_2 incubator at 37°C, and the slices were rotated alternately through air and medium at 1 rpm. Slices were used for assays at intervals, without a medium change.

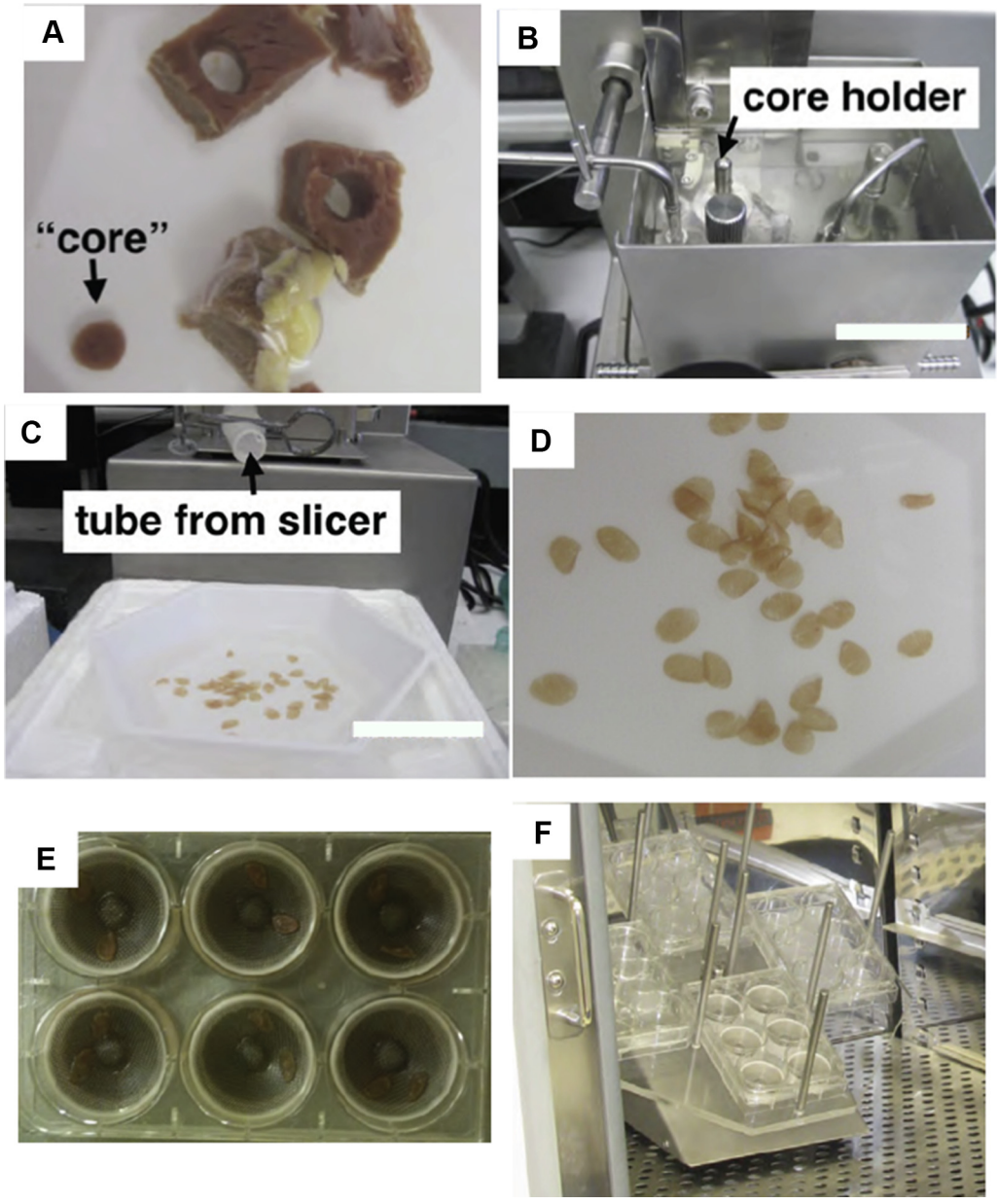
An average 1.5-cm thick LV free wall core cut into 250 μm slices provided a maximum of ~60 slices per core. A single LV provided multiple LV cores; 2 to 3 cores produced ~96 slices, which could be cultured in four 6-well culture trays. Additional trays could easily be added to increase throughput. After protocol optimization, slices usable for experiments were obtained in all hearts.

CHARACTERIZATION OF SLICE MORPHOLOGY, DIFFUSION, AND VIABILITY. **Figure 2A** is a slice schematic, illustrating key characteristics. The schematic indicates that the plane of slicing was parallel to the myocyte long axis; this is confirmed in **Figures 2B and 2C**. We estimated that each slice 8 mm diameter and 250 μm thick contained approximately 356,000 myocytes. This estimate is based on measurements of myocyte cross sectional area and length in the slices, to obtain an average volume 26,500 μm^3, within the range found in prior studied of human myocytes (15), and assumes that 25% of the slice is extracellular volume (16,17). Thus the total number of myocytes studied in an experiment with 96 slices was about 34 million. **Figure 2A** also indicates that the average slice wet weight was 8 mg, and the protein amount by Bradford assay was 2 mg.

To assess slice morphology, we did immunohistochemistry for β-MyHC, the main human myosin. **Figure 2B** shows that slices after 2 d culture had well aligned sarcomeres. Some wrinkling was evident, as expected for unloaded myocardium. Supplemental Figure 1 illustrates slice histology by hematoxylin and eosin staining.

A slice thickness of 250 μm was the best for both ease of cutting and rapid diffusion into the center of the slice, to avoid hypoxia. To measure diffusion, we took advantage of the red autofluorescence of doxorubicin, which is transported into cell nuclei (18). **Figure 2C** shows that doxorubicin was seen in myocyte nuclei at the center of the slice within 1 min,

FIGURE 1 Slice Generation and Culture

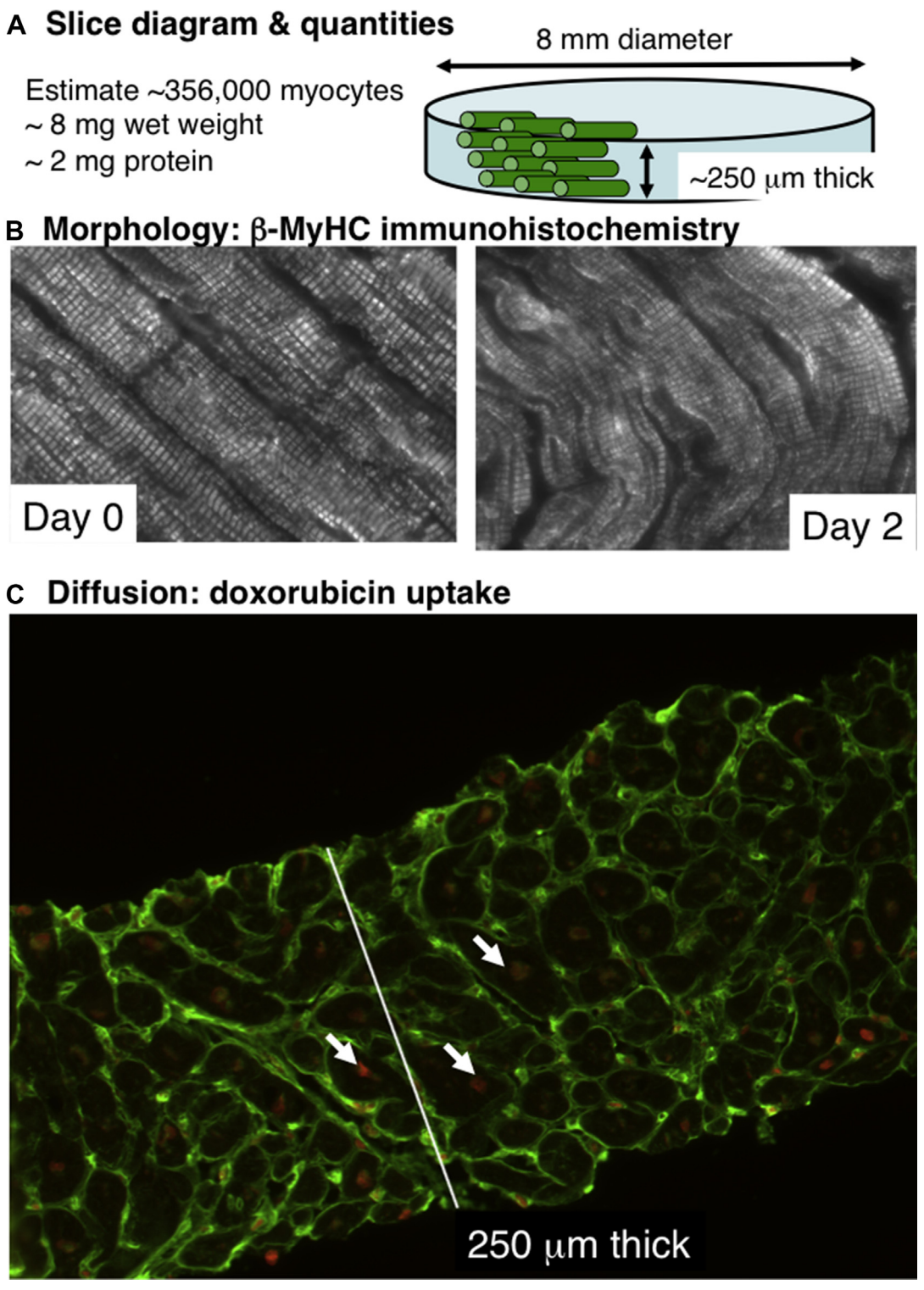


(A) A coring tool is used to punch an 8-mm diameter "core" from pieces of left ventricular free wall, perpendicular to myofiber orientation. **(B)** The core is trimmed, imbedded in agarose, and placed in a holder of the Krumdieck slicer, submerged in cold slicing buffer. **(C)** The core is passed across a razor blade at the bottom of the core holder to cut slices, and cold buffer delivers them into a receiving dish. **(D)** Slices "curl" slightly after cutting. **(E)** Two to 4 slices are placed on a titanium mesh in each well of a culture tray. **(F)** Four or more trays are placed on an inclined rotator in the incubator, so that slices rotate through air and medium interfaces.

indicating rapid diffusion. Figure 2C also shows that the top and bottom surfaces of the slice have mostly intact myocytes.

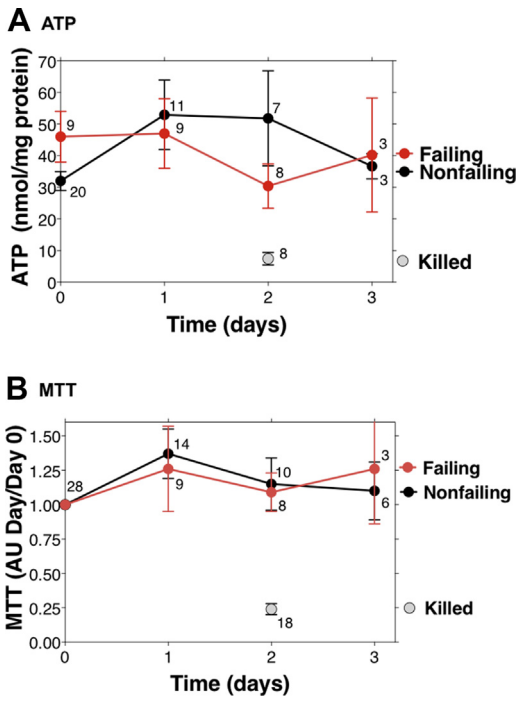
We used 2 assays to monitor slice viability, ATP and MTT. ATP on day 0, immediately after cutting, was 32 ± 3 nmol/mg protein in slices from nonfailing hearts (n = 20), and 46 ± 8 in slices from failing hearts (n = 9, p = 0.13 Student unpaired *t* test). The ATP level for nonfailing agrees extremely well with human heart studies (mean 31 nmol/mg, range 23 to

FIGURE 2 Slice Characteristics



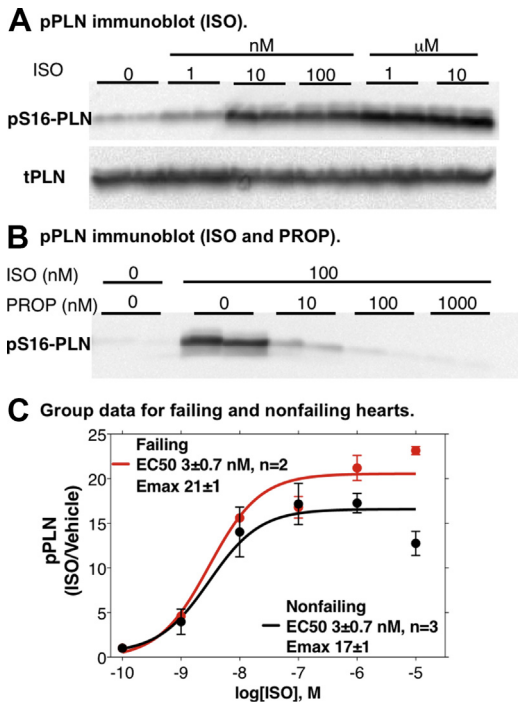
Slices are cut parallel to the myocyte long axis. **(A)** Slice dimensions, measured wet weight and protein, and estimated myocyte number (based on average volume 26,500 μm^3). **(B)** Beta-myosin heavy chain (β-MyHC) immunohistochemistry in a section tangential to a nonfailing slice shows well-aligned sarcomeres, with some wrinkling by culture day 2, likely caused by contraction. **(C)** A nonfailing slice cross section stained with wheat germ agglutinin shows doxorubicin (red autofluorescence) in myocyte nuclei in the center of the slice within 1 min of incubation, and clean top and bottom surfaces.

FIGURE 3 Human Myocardial Slices Are Viable for at Least 3 Days in Culture



Left ventricular myocardial slices were cultured as in the Methods section for the number of days indicated. Day 0 indicates slices immediately after cutting. As a negative control, slices were killed by prolonged anoxia, H_2O_2, or methanol. Adenosine triphosphate (ATP) **(A)** and MTT **(B)** were measured in duplicate slices from each heart. ATP duplicates agreed within an average 20%; and MTT, within an average 9%. MTT values in arbitrary units (AU) per mg after day 0 were normalized to the MTT value on day 0 in the same experiment. Numbers are the numbers of hearts; values are mean ± SE. There are no significant differences with time or between nonfailing and failing (regular 2-way analysis of variance: ATP $p = 0.23, 0.77, 0.54$ for interaction, group, and time, respectively; MTT $p = 0.91, 0.99$, and 0.44 for interaction, group, and time, respectively).

FIGURE 4 β-Adrenergic Activation of PLN in Human Heart Slices



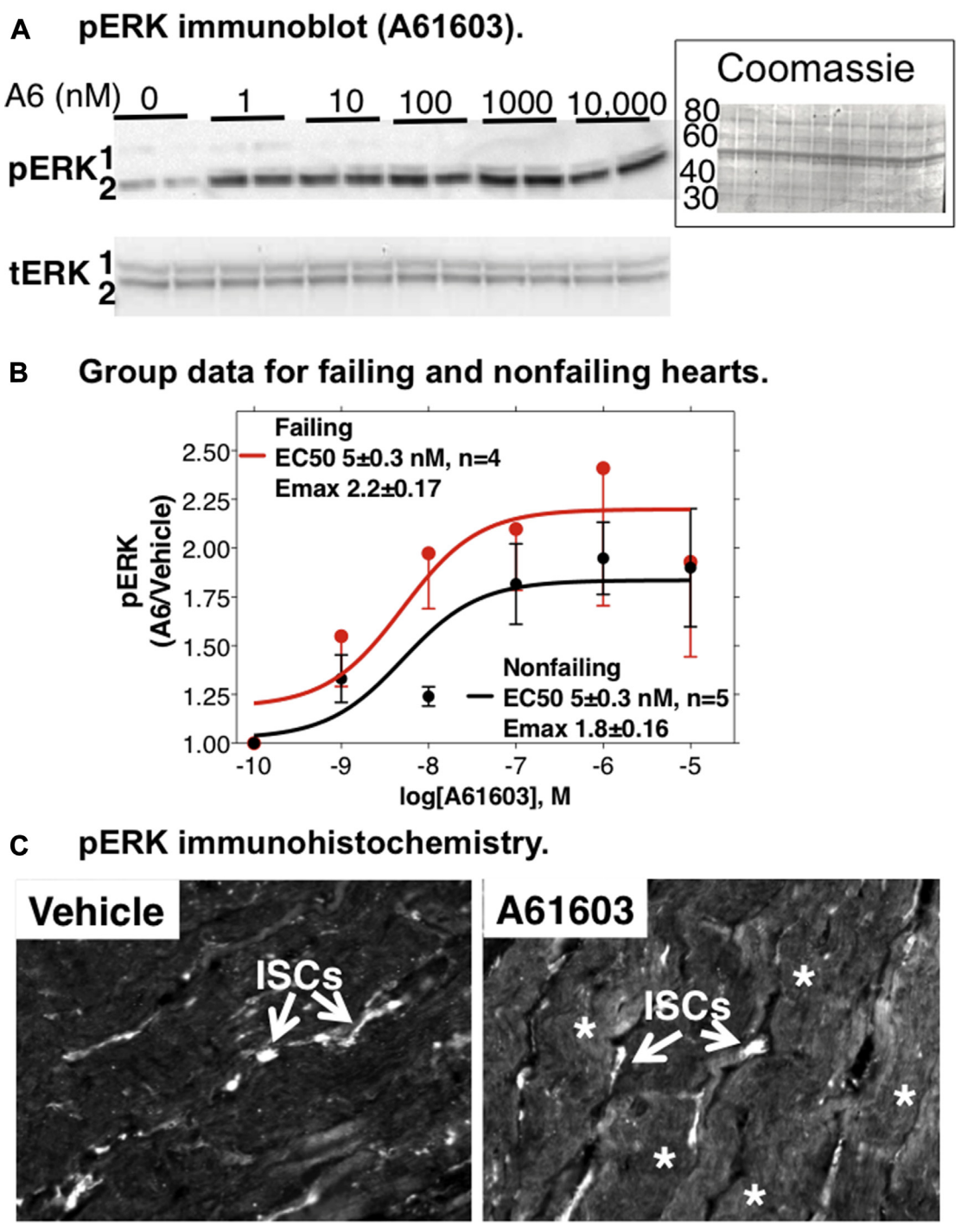
After 1 day in culture, duplicate left ventricular myocardial slices were treated for 15 min with varying doses of the β-adrenergic agonist isoproterenol (ISO), without or with the nonselective β-antagonist propranolol (PROP). Phosphorylation of phospholamban (PLN) at S16 was measured by immunoblot. **A** and **B** are from a nonfailing heart. **(A)** ISO doses. A blot of total PLN (tPLN) shows equal loading. **(B)** PROP inhibits ISO. **(C)** Concentration-response relationships for the indicated number of hearts, with duplicate slices for each heart at each dose; mean ± SE. The maximum effect values (Emax) for both failing (21) and nonfailing (17) are different from 1.0 ($p < 0.05$ by confidence limits). Half-maximal effective concentration (EC50) and Emax values for failing and nonfailing do not differ by curve fitting. These failing patients were treated with a left ventricular assist device.

38 nmol/mg, n = 5) (19-23). The ATP level in failing slices was higher than that seen in failing hearts in the same previous 5 studies (mean 25 nmol/mg, range 20-33). Figure 3A shows that slice ATP did not change significantly over 3 days in culture, in nonfailing or failing heart slices. Figure 3B shows the MTT assay for mitochondrial dehydrogenase activity was also not changed significantly over time.

Together, these data indicated good slice morphology, diffusion, and viability over at least 3 days, ample time to do experiments.

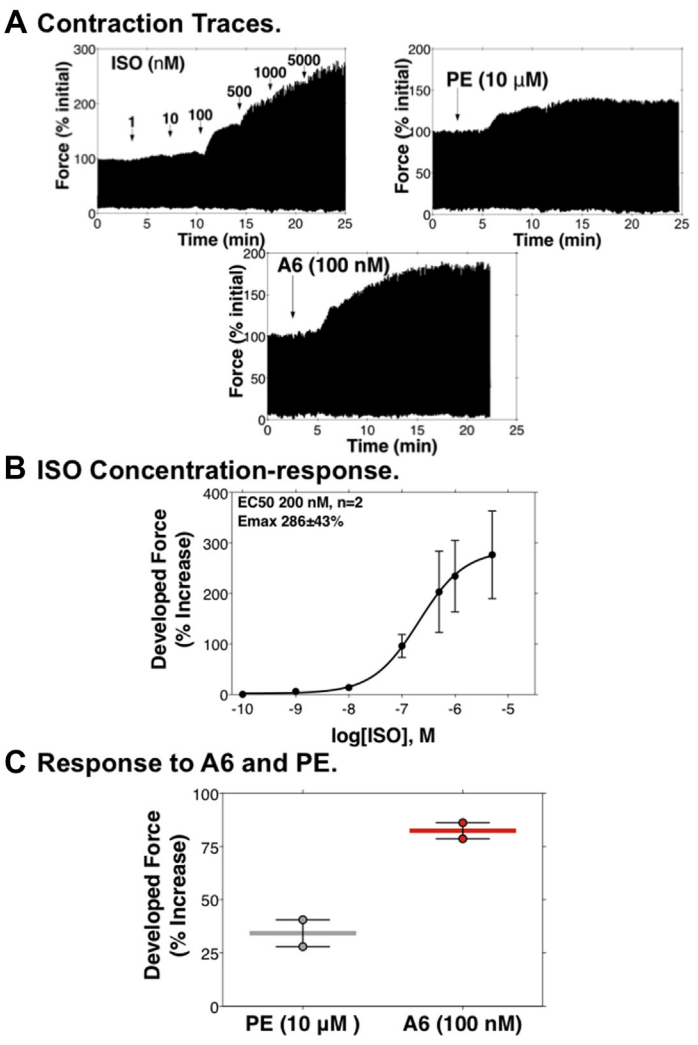
β-AR SIGNALING TO PLN. As a first test of signaling in the slice model, we sought to determine whether we could detect by immunoblot the expected β-AR stimulation of PLN phosphorylation. Duplicate slices after 1 day in culture were treated for 15 min with varying concentrations of the nonselective β-AR agonist ISO, in the absence or presence of the nonselective antagonist propranolol. Slices were rinsed and frozen in liquid nitrogen, then proteins were extracted in SDS with protease and phosphatase inhibitors, and used in immunoblot. Figure 4A shows that ISO stimulated robust phosphorylation of the protein kinase A target site serine 16. Figure 4B shows that the ISO effect was inhibited by a low concentration of propranolol, confirming β-AR dependence. Figure 4C shows concentration-response data. In

FIGURE 5 α1A-AR Activation of ERK in Human Heart Slices



After 1 day in culture, duplicate left ventricular myocardial slices were treated for 15 min with varying doses of the alpha-1A-adrenergic receptor (α1A-AR) agonist A61603 (A6). ERK dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2) was measured by immunoblot. **(A)** pERK immunoblot with varying A61603 doses in a nonfailing slice; a Coomassie-stained gel and a blot of total ERK (tERK) show equal loading. **(B)** Concentration-response relationships for total pERK in the indicated number of failing and nonfailing hearts, with duplicate slices for each heart at each dose; mean $\pm$ SE. Emax values for both failing and nonfailing are different from 1.0 ($p < 0.05$ by confidence limits). Failing and nonfailing Emax and EC50 do not differ by curve fitting. Three failing patients were treated with a left ventricular assist device, and 1 with carvedilol. **(C)** pERK immunohistochemistry in failing slices shows pERK in interstitial cells (ISCs) with both vehicle and A61603, and diffusely increased pERK in myocyte cytoplasm with A61603 1 μmol/l 15 min (e.g., **asterisks**). Abbreviations as in Figure 5.

FIGURE 6 The α1A-AR Stimulates a Positive Inotropic Effect in Human Myocardium



Strips ~ 0.12 mm^2 were cut from left ventricular slices of nonfailing hearts on culture day 1 and paced at 0.2 Hz and 37° C. ISO, phenylephrine (PE), and A6 were added as indicated. Data are from 2 hearts. **(A)** Examples of original contraction traces. **(B)** ISO concentration-response relationship for the increase in developed force (systolic − diastolic). Baseline systolic force was 1.4 millinewtons (mN)/mm^2; diastolic force was 0.8 mN/mm^2 and did not change with ISO. The increase in force is significantly different from 100% ($p < 0.05$ by confidence limits). **(C)** The increase in developed force with A6 and PE. Abbreviations as in Figure 5.

slices from both nonfailing and failing hearts, ISO stimulated serine 16 pPLN with a very low half-maximal effective concentration (EC50) (3 nmol/l) and a robust maximum (17- to 21-fold). These failing hearts were treated with an LVAD, perhaps explaining the preserved β-AR signaling.

α1A-AR SIGNALING TO ERK. To test α1A-AR signaling, we treated slices after 1 day in culture with the highly selective α1A-AR agonist A61603. The α1A-AR activates ERK in myocytes, and ERK activation is required for cardioprotective effects of the α1A-AR in mouse ventricular myocytes (24). A61603 is inactive when the α1A-AR is knocked out (25); and A61603 does not activate ERK in α1A KO myocytes (Myagmar and Simpson unpublished data, 2016). We quantified ERK activation by immunoblot for dual threonine-tyrosine phosphorylation of ERK1/2. Figure 5A shows a marked increase in pERK with A61603 as low as 1 nmol/l for 15 min. Group data for failing and nonfailing heart slices in Figure 5B indicate in both a maximum 1.8- to 2.2-fold increase in pERK, with an EC50 5 nmol/l ($n = 4$ to 5). Failing and nonfailing concentration-response curves did not differ.

Whereas PLN is myocyte specific, ERK is present in myocytes and nonmyocytes, and the latter contribute to ERK phosphorylation in diseased heart (26). To examine whether the increase in pERK by immunoblot was localized to myocytes, we did pERK immunohistochemistry on frozen sections after 15 min of treatment with A61603. Figure 5C shows bright pERK fluorescence in interstitial cells in slices treated with vehicle or A61603, and a clear increase in pERK in myocyte cytoplasm with A61603, indicating ERK activation in myocytes.

β- AND α1-AR STIMULATED CONTRACTION. To test contraction, we cut small strips from the slices, and mounted them in vitro, paced at 0.2 Hz and 37°C in medium with 1.8 mmol/l calcium. Slices assigned to contraction were cultured with BDM, but not blebbistatin, because the latter had slow washout.

A maximum dose of the subtype-nonselective agonist phenylephrine (PE) (10 μmol/l) or a maximum dose of the α1A-subtype-selective agonist A61603 (100 nmol/l) activated α1-ARs; acute inotropic responses were quantified when contraction force stabilized, typically ~20 min after addition. Varying doses of the subtype-nonselective agonist ISO activated β-ARs, and acute responses were measured ~150 to 200 s after ISO addition, when force reached a plateau.

Figure 6A illustrates with raw contraction traces that ISO, PE, and A61603 each had a positive inotropic effect in the strips cut from the slices. Figure 6B has summary data showing that ISO increased developed force by ~275%, with an EC50 225 nmol/l. Maximum systolic force was low, ~2 mN/mm^2, most likely because myocytes are oriented in different directions from top to bottom through the slice. A61603 100 nmol/l increased developed force by 82%, one-third as much as ISO (Figure 6B). The nonselective α1-AR agonist PE increased developed force by only 34%,

perhaps because of concomitant negative inotropic effects of the α1B-AR, which is also stimulated by PE, but not by A61603.

VIRAL TRANSDUCTION OF HUMAN MYOCARDIAL SLICES. To test whether slices could be used for transduction with virus, we did a pilot study with 1 heart. Slices were infected with adenovirus containing PKD1 with an N-terminal GFP tag. Supplemental Figure 2 shows GFP immunoreactivity in every myocyte exposed to 250 plaque-forming units of virus, the lowest amount tested, indicating successful transduction.

TABLE 1 Comparison of Human Myocardial Slices vs. Isolated or Cultured Ventricular Myocytes

Slice Advantages	Slice Disadvantages
Much easier	Cell heterogeneity (need cell-specific assays)
More reproducible	Used less (fewer comparison data)
Much higher throughput (estimated 356,000 myocytes per slice, up to 96 slices with total 34 million myocytes per four 6-well culture trays; multiple sets of 4 trays per culture incubation unit) vs. 1 to 2 million cells per isolation	Initial expense of equipment
Unbiased (no selection for healthier cells)	
More physiological (paracrine and autocrine signaling preserved)	
Efficient use of hearts (more cells per heart)	
Enables biochemistry, histology, and physiology	

DISCUSSION

We developed and validated a myocardial slice model to study signaling in the human heart. This simple, reproducible, high throughput approach generates a large number of slices from LV myocardium that can be used for biochemistry, contraction, and viral transduction.

Myocyte morphology and viability are maintained over at least 3 days, ample time to do assays. This model could be useful for translational studies, to test whether signaling molecules identified in animal studies are active in human myocytes. As proof of concept, we show for the first time that the α1A-AR mediates ERK activation and a positive inotropic effect in human LV myocardium.

Table 1 compares the advantages and disadvantages of this new slice model, in comparison with isolated and cultured ventricular myocytes. The advantages with respect to other tissue preparations, for example, trabeculae (as mentioned in the Introduction section), include reproducibility, throughput, and validation in biochemical and other assays. With this slice model, or any tissue preparation, it is required to show that observed signaling is in myocytes. We studied pPLN, which is myocyte specific, and pERK, which is present in all cell types. To show ERK activation in myocytes, we used immunohistochemistry, and the immunohistochemistry approach should be useful in most cases. This slice model also requires an initial equipment outlay, for the coring press, Krumdieck slicer, and culture incubation unit. We consider these important to the success of the protocol.

Other aspects of the protocol we consider especially important for optimum slices include heart perfusion and transport in cold cardioplegia, with total time from explant to slicing $< \sim 4$ h; slice cutting parallel to the myocyte long axis; BDM ± blebbistatin in slice preparation and culture; and slice thickness 250 μm. Slice viability was improved by slow calcium reintroduction and adding serum to the culture medium. The detailed protocol we provide in the Methods section should enable replication in other laboratories.

In contrast with prior human myocardial slice reports (11–13), our report is the first used to study biochemical signaling, includes the most hearts by far, studies both failing and nonfailing, and presents a detailed protocol. Only 1 other study used explants, rather than biopsies, and included only 3 hearts (13). One report studied electrophysiology and contraction in slices cut from LV outflow tract biopsies of patients having valve replacement; 4 to 20 slices were obtained from each patient (12). The slices were viable as long as 28 days by MTT assay, but the fibroblast marker vimentin was increased by 5-fold at day 4 and by 18-fold by day 28 (12), raising the possibility that some of the MTT signal at 28 days was from proliferating fibroblasts. In addition, several markers of myocyte differentiation were reduced with time, including myosin light chain 2 mRNA, visible cross striations, contractility, and action potentials (12). These changes are consistent with the observation that fibroblast overgrowth can impair myocyte differentiation (27). To minimize such time-dependent changes, we advocate using the slices over the first few days, when the slices are most stable. Using this model, we were able to discover novel translational biology.

Specifically, the model enabled us to show for the first time to our knowledge that the α1A-AR is active in signaling and contraction in human LV myocardium, by measuring the effects of the highly selective α1A-AR agonist A61603. The α1A-AR is present at very low levels in human LV (as mentioned in the Introduction section), and it was unknown whether this low level could mediate signaling. The robust ERK activation in failing and nonfailing slices is notable, as well as a positive

inotropic effect that is one-third the magnitude of ISO in nonfailing slices. The α1A-AR via ERK activation is protective in mouse myocytes (24), and A61603 can prevent apoptosis in the mouse heart in vivo (28). The present data support the possibility that the protective effect of α1A activation in mouse might extend to the human heart.

STUDY LIMITATIONS. A caveat is that signaling ex vivo might not occur in vivo. However, approaches to study signaling in the human myocardium in vivo are lacking.

CONCLUSIONS

In summary, mouse models are valuable for investigating molecular mechanisms of cardiac disease and suggesting potential therapeutic targets. However, fundamental differences between mouse and human physiology raise uncertainty regarding the human disease relevance of findings in mice. Therefore, studies in human myocardium are essential but challenging due in part to their difficulty and low throughput. This model addresses an important need in describing a simple, high throughput system to study signaling in human myocardium, and reveals for the first that the α1A-AR is functional in the human heart.

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REPRINT REQUESTS AND CORRESPONDENCE: Dr. Paul C. Simpson, VA Medical Center (111-C-8) 4150 Clement Street, San Francisco, California 94121. E-mail: paul.simpson@ucsf.edu.

PERSPECTIVES

TRANSLATIONAL OUTLOOK: Basic research is identifying multiple potential targets for drugs to treat heart failure and myocardial disease, but translation to clinical trials is very difficult. Species differences exist between humans and mice and other pre-clinical models. Evidence that potential targets are functional in human myocardium might facilitate translation, but models to test signaling in human myocardium are limited. We describe a simple, reproducible, high-throughput human myocardial slice model to study signaling. As proof of usefulness, we show for the first time that a very low abundance cardioprotective receptor, the alpha-1A-adrenergic receptor, is functional in nonfailing and failing human myocardium.

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APPENDIX For supplemental figures, please see the supplemental appendix of this article.