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Ryanodine Receptor Calcium Leak in Circulating B-Lymphocytes as a Biomarker in Heart Failure

Alexander Kushnir, MD, PhD
Gaetano Santulli, MD, PhD
Steven R. Reiken, PhD
Ellie Coromilas, MD
Sarah J. Godfrey, BA
Danielle L. Brunjes, PhD
Paolo C. Colombo, MD
Melana Yuzefpolskaya, MD
Seth I. Sokol, MD
Richard N. Kitsis, MD*
Andrew R. Marks, MD*

BACKGROUND: Advances in congestive heart failure (CHF) management depend on biomarkers for monitoring disease progression and therapeutic response. During systole, intracellular Ca^{2+} is released from the sarcoplasmic reticulum into the cytoplasm through type-2 ryanodine receptor/Ca^{2+} release channels. In CHF, chronically elevated circulating catecholamine levels cause pathological remodeling of type-2 ryanodine receptor/Ca^{2+} release channels resulting in diastolic sarcoplasmic reticulum Ca^{2+} leak and decreased myocardial contractility. Similarly, skeletal muscle contraction requires sarcoplasmic reticulum Ca^{2+} release through type-1 ryanodine receptors (RyR1), and chronically elevated catecholamine levels in CHF cause RyR1-mediated sarcoplasmic reticulum Ca^{2+} leak, contributing to myopathy and weakness. Circulating B-lymphocytes express RyR1 and catecholamine-responsive signaling cascades, making them a potential surrogate for defects in intracellular Ca^{2+} handling because of leaky RyR channels in CHF.

METHODS: Whole blood was collected from patients with CHF, CHF following left-ventricular assist device implant, and controls. Blood was also collected from mice with ischemic CHF, ischemic CHF+S107 (a drug that specifically reduces RyR channel Ca^{2+} leak), and wild-type controls. Channel macromolecular complex was assessed by immunostaining RyR1 immunoprecipitated from lymphocyte-enriched preparations. RyR1 Ca^{2+} leak was assessed using flow cytometry to measure Ca^{2+} fluorescence in B-lymphocytes in the absence and presence of RyR1 agonists that empty RyR1 Ca^{2+} stores within the endoplasmic reticulum.

RESULTS: Circulating B-lymphocytes from humans and mice with CHF exhibited remodeled RyR1 and decreased endoplasmic reticulum Ca^{2+} stores, consistent with chronic intracellular Ca^{2+} leak. This Ca^{2+} leak correlated with circulating catecholamine levels. The intracellular Ca^{2+} leak was significantly reduced in mice treated with the Rycal S107. Patients with CHF treated with left-ventricular assist devices exhibited a heterogeneous response.

CONCLUSIONS: In CHF, B-lymphocytes exhibit remodeled leaky RyR1 channels and decreased endoplasmic reticulum Ca^{2+} stores consistent with chronic intracellular Ca^{2+} leak. RyR1-mediated Ca^{2+} leak in B-lymphocytes assessed using flow cytometry provides a surrogate measure of intracellular Ca^{2+} handling and systemic sympathetic burden, presenting a novel biomarker for monitoring response to pharmacological and mechanical CHF therapy.

*Drs Kitsis and Marks contributed equally.

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Clinical Perspective

What Is New?

- Systemic neurohormonal activation in heart failure causes dysfunctional intracellular calcium handling in cardiomyocytes, leading to disease progression.
- It is not feasible to assess and monitor this in patients.
- We demonstrate in humans and mice with heart failure that circulating B-lymphocytes exhibit dysfunctional intracellular calcium handling in a manner similar to what is observed in cardiomyocytes.
- A subgroup of patients with heart failure following left-ventricular assist device implant exhibited improved intracellular calcium handling.
- This finding was assessed using flow cytometry, a readily available clinical laboratory technique.

What Are the Clinical Implications?

- Testing intracellular calcium handling in circulating B-lymphocytes is a potential novel biomarker for myocardial dysfunction and response to therapy in patients with heart failure and may help identify patients with myocardial recovery following left-ventricular assist device implant.

Congestive heart failure (CHF) is a leading cause of hospitalization and mortality in the United States.[1] Left ventricular assist devices (LVAD) are indicated for patients with end-stage heart failure as either a bridge-to-transplant or destination therapy.[2] However, with 1-year survival after implant reaching 80%, there is a greater potential for ventricular reconditioning and pump explant.[3] Biomarkers for pump recovery and response to CHF therapies in general are needed to help guide device and pharmacological management.

Elevated circulating plasma norepinephrine (NE) levels are associated with CHF progression and mortality,[4,5] and reduced NE levels are observed in the setting of CHF therapy.[6,7] However, the complexities associated with handling and processing NE assays limit the clinical utility of direct serum measurements.[8] Novel approaches to assaying the effect of sympathetic activity in patients with heart failure are needed.

NE binds to β-adrenergic receptors on myocytes in both the heart and skeletal muscle, causing production of cyclic AMP, which activates protein kinase A (PKA)[9] as well as other protein kinases that phosphorylate targets in the skeletal muscle[10] and heart and regulate gene expression.[11] The type 2 ryanodine receptor (RyR2) is an intracellular, sarcoplasmic reticulum (SR)-based Ca^{2+} release channel responsible for providing the Ca^{2+} necessary for myocardial contraction.[12] In human myocardium, PKA phosphorylates (RyR2) at Ser2809 (Ser2808 in murine RyR2).[13–15] In skeletal muscle, PKA phosphorylates the type 1 ryanodine receptor (RyR1) at Ser2843 (Ser2844 in murine RyR1).[16,17] Under physiological conditions, PKA phosphorylation of RyR2 in the heart and RyR1 in skeletal muscle contribute to modulating SR Ca^{2+} release and muscle contractility.[13,16,17] RyR channel phosphorylation by CaMKII also plays a role in rate-related increase in cardiac contractility,[18] although nonphosphorylation mechanisms have also been described.[19]

In CHF, persistently elevated circulating NE levels cause chronic pathological phosphorylation of RyR2[14,20] and RyR1.[17,21] This results in remodeling of the channel macromolecular complex, including nitrosylation/oxidation, dissociation of the phosphodiesterase PDE4D3 (which exacerbates PKA phosphorylation), and decreased binding of the stabilizing subunit calstabin to the channel.[10,14,17,22–26] These events together cause localized Ca^{2+} sparks[27] and more generalized postrepolarization Ca^{2+} release events.[14,15,20,25,28,29] In the heart, this results in diastolic SR Ca^{2+} leak, decreased SR Ca^{2+} load, and reduced contractility and cardiac output.[20,30] Moreover, chronic SR Ca^{2+} leak via RyR2 channels causes mitochondrial Ca^{2+} overload, perpetuating metabolic dysfunction in failing hearts.[31] Additionally, the Ca^{2+} leak and resultant increase in cytosolic (Ca^{2+}) generate a transient inward current through Na^{+}/Ca^{2+} exchangers, which partially depolarizes the membrane, increasing the propensity for ectopic activity and arrhythmias.[32,33]

In skeletal muscle, leaky RyR1 in CHF contributes to muscle weakness.[21] None of the current treatments for CHF improves skeletal muscle function, including β-blockers. However, a novel experimental class of drugs called Rycals prevents stress-induced dissociation of the channel stabilizing subunit calstabin2 from RyR2 and calstabin1 from RyR1, thereby reducing RyR-mediated SR Ca^{2+} leak[21,28,34–36] and resulting in improved cardiac and skeletal muscle function.[21] S107, the Rycal drug used in the present study, is in the same chemical class as the drug currently in clinical testing and shares the same mechanism of action: fixing leaky RyR channels.

In addition to its pivotal role in muscle contraction, intracellular Ca^{2+} release and handling contributes to B-lymphocyte activation during an immune response.[37] Store-operated Ca^{2+} entry through the plasma membrane is a major source of intracellular Ca^{2+} during an immune response. This is triggered by emptying the ER Ca^{2+} stores by activation of inositol 1,4,5-trisphosphate receptors, which are regulated by phosphorylation in lymphocytes.[37–42] RyR1 expression has been observed in B-lymphocytes.[43,44] Although RyR1 Ca^{2+} store contribution to immune function has not been elucidated, it can be assessed using flow cytometry.[45] In addition to expressing RyR1, B-lymphocytes express the components of the β-adrenergic cascade necessary for activating PKA in response to circulating NE.[46] In this study, we investigated whether the elevated NE levels in CHF cause pathological RyR1 remodeling and Ca^{2+}

leak in circulating B-lymphocytes in humans and mice and whether this could serve as a biomarker for S107 therapy and response to LVAD mechanical support.

METHODS

All reagents and materials are available on request.

Mouse CHF Model and Blood Sample Collection

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University and according to National Institutes of Health guidelines. A postinfarct mouse model of CHF was utilized. Studies were conducted over a 4-month period. Permanent occlusion of the mid left anterior descending (LAD) coronary artery was performed in 4-month-old male C57BL/6 mice as described.[21,25] Mid LAD, as opposed to proximal LAD, was chosen to induce mild to moderate myocardial dysfunction while limiting mortality. Male mice were utilized to limit any potential effect of hormonal cycles.[47] Briefly, mice were anesthetized, intubated, and mechanically ventilated under isoflurane anesthesia with buprenorphine subcutaneous 0.05 mg/kg for analgesia. A left thoracotomy was performed via the third intercostal space, and muscles and pericardium were carefully dissected. The LAD coronary artery was identified using a dissecting microscope and ligated using an 8-0 nonabsorbable suture. The intercostal space was closed using a 6-0 nonabsorbable suture and the skin with a 5-0 absorbable suture. Mice received 100% oxygen until wakening, after which the endotracheal tube was withdrawn. Animals were then given oxygen via nasal cone until full recovery of consciousness. Buprenorphine subcutaneous 0.05 mg/kg was provided every 12 hours for 2 to 3 days for postoperative analgesia. Mice were randomly assigned to 3 groups (n=10 each): CHF+vehicle, CHF+S107 (50 mg/kg/day in drinking water from time of surgery), and Sham (mice underwent the same surgical procedure except for LAD occlusion). After randomization, there were no readily detectable baseline differences between each group, and baseline cardiac function as determined by echocardiography was similar. Starting 8 weeks after surgery, blood was collected from the retro-orbital plexus into 1.5 mL tubes containing 7.5 ul heparin 1000 u/mL for experiments. Red blood cells were eliminated using red blood cell lysis buffer (BioLegend) per manufacturer's instructions. For biochemistry experiments, the lymphocyte enriched pellets were frozen and stored at −80°C. Tubes were labeled using a number system, and the experimenters were blinded to the contents. To ensure adequate cell counts, lymphocyte-enriched pellets from mice could be combined within their respective groups. Cardiac function was assessed by echocardiography using a Visualsonic Vevo 770 ultrasound equipped with a 30-MHz transducer applied to the chest wall. Fractional shortening was assessed in the M-mode.

Patient Enrollment and Blood Sample Collection

All human studies were performed according to protocols approved by the Institutional Review Boards at Albert Einstein College of Medicine and Columbia University–New York Presbyterian Hospital. Patients with CHF and controls were recruited from the inpatient and outpatient services at Jacobi Medical Center (JMC). Patients with CHF and CHF+LVAD and controls were recruited from the inpatient and outpatient services at Columbia University Medical Center–New York Presbyterian Hospital (CUMC). All subjects gave informed consent. When available, baseline characteristics, including age, sex, ejection fraction, New York Heart Association CHF class, etiology of CHF (ischemic versus nonischemic), hypertension, diabetes mellitus, hyperlipidemia, ACE inhibitor use, β-blocker use, blood pressure, and heart rate, were recorded. All patients using LVADs had their devices implanted for ≥9 months and were hemodynamically stable. Samples were deidentified before testing. For samples acquired at Jacobi Hospital, lymphocyte-enriched pellets were isolated from 7 to 8 mL of whole blood collected from patients using BD CPT vacutainers (containing premade Ficoll-Hypaque gradient) with Na-citrate as previously described.[48] In brief, the tubes were centrifuged at 1700 *g* for 15 minutes, and 1xHBSS was added to supernatant, final volume 15 mL. Samples were centrifuged at 300 *g* for 10 minutes. Pellets were washed twice with 10 mL HBSS and resuspended in HBSS. For samples acquired at CUMC, 2 to 4 mL of blood were collected using Li-heparin vacutainers, and red blood cells were eliminated using red blood cell lysis buffer (BioLegend) per manufacturer's instructions.

Biochemistry

Immunoprecipitation of RyR from lymphocyte-enriched cell lysates was performed using an RyR antibody (5029)[13,49,50] in 0.5 mL of a modified RIPA buffer (50 mM Tris-HCl pH 7.2, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na_3VO_4, 1% Triton-X100, and protease inhibitors) overnight at 4°C. RyR1 and RyR2 isoform-specific rabbit polyclonal antibodies were used to immunoprecipitate (1:200) specific RyR isoforms. These antibodies were generated against either the peptide sequence KPEFNNHKDYAQEK, corresponding to amino acids 1367 to 1380 of mouse cardiac RyR2 (also 1367–1380 of human RyR2), or the peptide sequence AEPDTDYENLRRS, corresponding to amino acids 1327 to 1339 of the mouse skeletal muscle RyR1 (also 1325–1337 of human RyR1).[18,25,51] Rabbit cardiac and skeletal muscle SR lysates were used as controls. The immune complexes were incubated with protein A Sepharose beads (Sigma) at 4°C for 1 hour, and the beads were washed 3 times with RIPA buffer. The immunoprecipitates were size-fractionated on SDS-PAGE gels (6% for RyR, 15% for calstabin) and transferred onto nitrocellulose membranes for 2 hours at 200 mA. Immunoblots were developed using the following primary antibodies: RyR 5029 (1:5000),[13] antiphospho-RyR1-pSer2843 (1:5,000),[13,17] and calstabin (FKBP12 C-19, 1:1000, Santa Cruz Biotechnology). To determine channel oxidation, the carbonyl groups on the protein side chains of immunoprecipitated RyR channels were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazone signal associated with RyR was determined using a specific 2,4-dinitrophenylhydrazone antibody, according to the manufacturer's instructions (Millipore).[52] All immunoblots were developed with the Odyssey system (LI-COR Biosciences) using IR-labeled mouse and rabbit IgG (1:10000 dilution)

secondary antibodies. For immunoblot analyses of SERCA2, phospholamban (PLB), and PLB phosphorylation at Serine16 (PLB-S16), lymphocyte pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 20 mM NaF, and protease inhibitors) and then size-fractionated on 15% SDS-PAGE gels, transferred onto nitrocellulose membranes, and stained with the following primary antibodies: SERCA2 1:1000 (Santa Cruz Biotech), total PLB1:1,000 (MA3-922; Thermo Scientific), phospho-specific PLB-Ser16 1:1,000 (20R-P121A; Fitzgerald), and GAPDH 1:1000 (Abcam). Experiments were performed by blinded individuals.

Norepinephrine Measurements

Blood samples were drawn into EDTA-coated BD vacutainers, and plasma was isolated by centrifugation for 15 minutes at 2000 *g*. Plasma was aliquoted and frozen at −80°C within 1 hour of draw. NE was measured using the epinephrine/norepinephrine ELISA kit following the manufacturer's instructions.

Flow Cytometry

In both the human and murine experiments, the washed lymphocyte-enriched pellets were suspended in 100 microliters HBSS containing a fluorescent-tagged CD19 antibody. Either phycoerythrin (human experiments: Abcam ab1168; mouse experiments: Abcam ab90753) or APC-tagged CD19 antibodies was used (Invitrogen, SJ25-C1), concentrations per manufacturer's recommendations. After a 10-minute incubation, 400 microliters of 1 micromolar Fluo-4 AM in 1x HBSS was added. The lymphocytes were allowed to load with Fluo-4 AM for 30 minutes, and then the pellets were washed twice with 1x HBSS to remove excess Fluo-4 AM and fluorescent antibody. Baseline fluorescence was analyzed using a Becton Dickinson LSR II flow cytometer. Side scatter and forward scatter light were used for gating the lymphocyte population. Crossover of phycoerythrin and Fluo-4 fluorescence was compensated using single fluorophore controls. Fluorescent measurements were acquired in the absence and presence of RyR agonists, either caffeine (50 mM) or 4-CmC (0.5 mM).

Statistics

Data are expressed as mean ±SEM. Two-tailed students *t* test or ANOVA with Tukey multiple-comparison test were used for calculating significance when applicable. χ-square test was used for calculating significance between patient groups. Quantification of immunoblots is expressed in arbitrary units. Sample size in the mouse study was calculated by assuming an absolute difference of 15% between groups with a standard deviation of 10%, with β error 20%, α error 5%, and accounting for possible deaths before experimentation. Mortality for mouse studies was plotted using Kaplan-Meyer survival curves, and statistical significance was calculated using Mantel–Cox log-rank test.

RESULTS

RyR1 in B-Lymphocytes Is Remodeled in Humans and Mice With CHF

Patients from 2 medical centers with and without CHF were recruited for a 1-time donation of whole blood. Patients ≥9 months after LVAD implant and hemodynamically stable were recruited from 1 medical center. There were significantly fewer women in the CHF and LVADs group compared with the normal group (Table). The EF in the LVAD group was significantly reduced compared with

Table. Baseline Characteristics of Patients Included in This Study

Baseline Characteristics	Normal (N=38)	Heart Failure (N=33)	Left Ventricular Assist Device (N=14)	*P*
Age	57±2	65±2	63±3	<0.05
Female (%)	53	30	7	<0.05
Ejection fraction	63±1	23±4	16±1	<0.05
New York Heart Association class, n (%)				
I	N/A	7 (21)	N/A	
II	N/A	8 (24)	N/A	
III	N/A	10 (30)	N/A	
IV	N/A	8 (24)	N/A	
Systolic blood pressure average	133±4	126±4	95±4	<0.05
Diastolic blood pressure average	74±2	69±2	69±3	NS
Heart rate average	77±3	81±3	79±4	NS
Angiotensin-converting enzyme/angiotensin receptor blocker, n (%)	7 (19)	26 (78)	5 (36)	<0.05
β-Blocker, n (%)	12 (33)	28 (85)	13 (93)	<0.05
Hypertension, n (%)	24 (67)	25 (75)	11 (79)	NS
Diabetes mellitus, n (%)	11 (31)	16 (48)	4 (29)	<0.05
Hyperlipidemia, n (%)	14 (42)	18 (54)	8 (57)	NS
Ischemic, n (%)	N/A	15 (45)	8 (57)	<0.05

Samples from patients were used for either flow cytometry or biochemistry. N/A indicates not applicable; and NS, no significance.

both normal patients and those with CHF. About half of patients with CHF and LVAD had ischemic cardiomyopathy, and all 4 New York Heart Association CHF classes were represented. Although β-blocker and ACE inhibitor/angiotensin receptor blocker use was significantly higher in the CHF group compared with the normal group, there were no significant differences in the heart rates or blood pressures between the 2 groups. However, systolic blood pressure was significantly lower in the LVAD group compared with both the CHF and normal groups (Table).

Immunoprecipitation using isoform-specific RyR1 and RyR2 antibodies demonstrated that RyR1 but not RyR2 is expressed in circulating peripheral lymphocytes (Figure 1A), consistent with previous reports.[43] RyR1 from patients with CHF exhibited increased PKA phosphorylation at Ser2843, oxidation, and depletion of calstabin1 compared with control patients (Figure 1B and 1D through 1F; see also Figure I in the online-only Data Supplement). These findings are consistent with previous reports that RyR2 from human myocardium[14,34] and RyR1 from human[53] and canine[17] skeletal muscle are PKA phosphorylated and depleted of calstabin2 and calstabin1, respectively, in CHF. Levels of SERCA2, total PLB, and PKA phosphorylation of PLB at Ser16 were unchanged in the human lymphocyte CHF samples (Figure 1C and 1G). These data suggest that RyR1 and SERCA2 in B-lymphocytes are affected differently in CHF, and that RyR1 may exhibit increased opening and SR Ca^{2+} release without a corresponding increase in SR Ca^{2+} uptake via SERCA2.

To validate that these results are specific to CHF, ischemic CHF was induced in mice by LAD occlusion as previously described.[25] The CHF treatment group received S107, a drug that reduces Ca^{2+} leak through dysfunctional RyR1[51] via drinking water.[35] RyR1 was immunoprecipitated from lymphocyte-enriched pellets (Figure 2A). RyR1 from CHF mice exhibited increased PKA phosphorylation at Ser2844, oxidation, and depletion of calstabin1 compared with sham mice (Figure 2B through 2D; see also Figure II in the online-only Data Supplement). Treatment with S107 did not affect PKA phosphorylation or oxidation of RyR1 but increased the level of calstabin1 that coimmunoprecipitates with the channel (ie, is part of the channel macromolecular complex) as previously reported.[22] These findings support our observations in RyR1 isolated from human lymphocytes and are also consistent with previous reports that RyR2 and RyR1[21] are PKA phosphorylated, oxidized, and depleted of calstabin in mice with CHF.[20,26]

RyR1 in B-Lymphocytes Exhibits Altered Ca^{2+} Handling in Humans and Mice With CHF

In skeletal muscle, these biochemical changes in RyR1 (phosphorylation, depletion of clastabin1) are associated with channel dysfunction and Ca^{2+} leak.[21] Flow cytometry has been previously used to assess RyR1 Ca^{2+} handling in B-lymphocytes from patients with malignant hyperthermia, a condition caused by dysfunctional RyR1.[45] We used flow cytometry to assess ER Ca^{2+} load

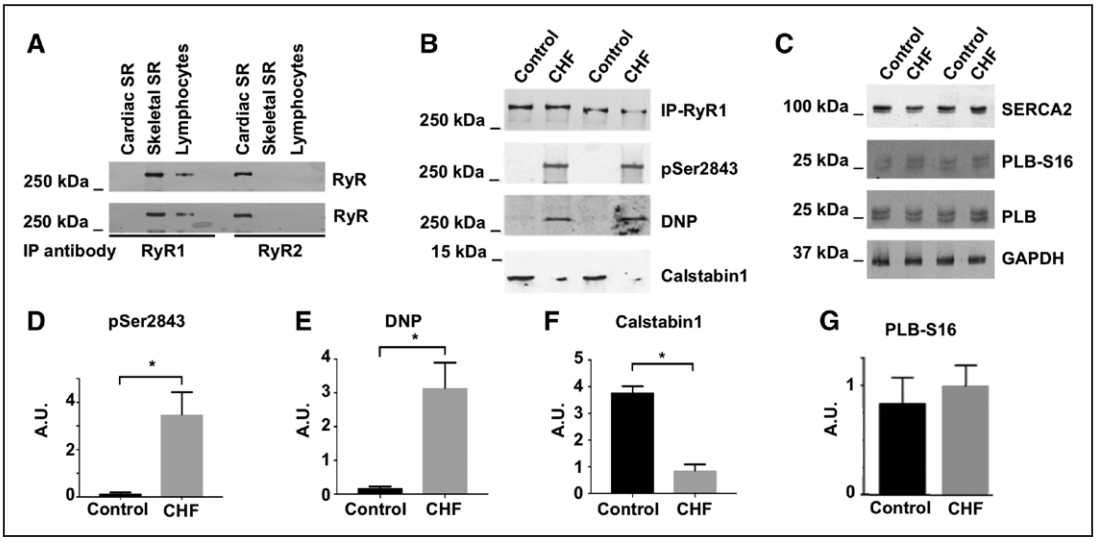

Figure 1. RyR1 expressed in lymphocyte-enriched cell population is pathologically remodeled in patients with CHF.
A, RyR1 is immunoprecipitated from a lymphocyte-enriched cell population isolated from normal patients using RyR1- but not RyR2-specific antibodies. Experiment performed on lymphocytes isolated from 2 patients. **B**, RyR1 immunoprecipitated from 2 patients with CHF exhibits increased PKA phosphorylation at Ser2843 and oxidation (DNP), as well as depletion of calstabin1 compared with 2 control patients. **C**, SERCA2 and PLB expression levels and PKA phosphorylation of PLB at Ser16 are unchanged in lymphocytes from 2 patients with CHF and 2 control patients. **D**–**F**, Quantification of data from 6 patients with CHF and normal patients (includes data from Figure I in the online-only Data Supplement). Results are normalized to total RyR1 levels and expressed in arbitrary units. **G**, Quantification of phosphorylation of PLB at Ser16 normalized to total PLB, and GAPDH levels expressed in arbitrary units from Western blot in (**C**). CHF indicates congestive heart failure; DNP, 2,4-dinitrophenylhydrazone; PKA, protein kinase A; PLB, phospholamban; RyR1, type 1 ryanodine receptor; RyR2, type 2 ryanodine receptor; and SR, sarcoplasmic reticulum. *P<0.05.

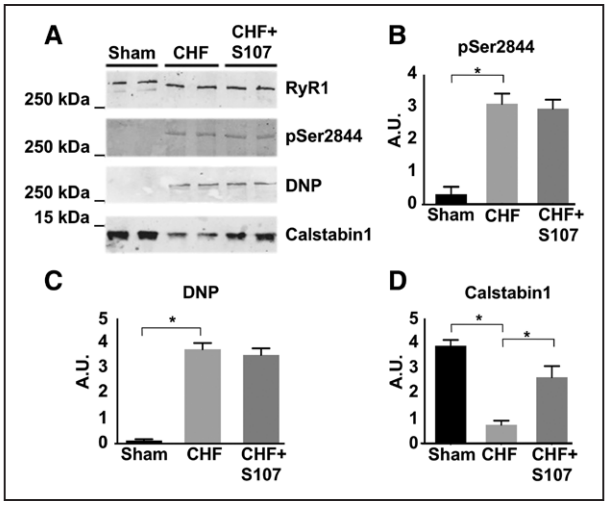

Figure 2. Pathological remodeling of RyR1 in lymphocyte-enriched cell population from mice with CHF and reversal with S107.
A, RyR1 immunoprecipitated from murine lymphocyte-enriched cell population exhibits increased PKA phosphorylation at Ser2844, oxidation, and depletion of calstabin1 compared with wild-type mice. S107 inhibits the depletion of calstabin1 from the RyR1 complex without affecting PKA phosphorylation or oxidation levels. Experiments were performed from samples isolated from 4 mice in each group. **B**–**D**, Quantification of Western blot data (includes data from Figure II in the online-only Data Supplement) expressed in arbitrary units. CHF indicates congestive heart failure; DNP, 2,4-dinitrophenylhydrazone; PKA, protein kinase A; and RyR1, type 1 ryanodine receptor. *P<0.05.

in B-lymphocytes as an indicator of chronic RyR1 Ca^{2+} leak resulting from exposure to elevated circulating catecholamine levels. B-lymphocytes were loaded with Fluo-4 AM, a cytosolic-based fluorescent Ca^{2+} indicator. Baseline cytosolic Ca^{2+} fluorescence was measured. Afterward an RyR1 agonist that induces release of RyR1-mediated ER Ca^{2+} stores, either caffeine or 4-CmC,[45,54] was added to the cells, and then Ca^{2+} fluorescence was remeasured. The % change of mean cytosolic Ca^{2+} fluorescence of CD-19+ cells after the addition of either RyR1 agonist was calculated. Cells with reduced ER Ca^{2+} load secondary to chronic RyR1 Ca^{2+} leak would be expected to have a smaller % increase in cytosolic Ca^{2+} after the addition of either RyR1 agonist compared with cells without chronic RyR1-mediated ER Ca^{2+} leak.

A mouse model of ischemic CHF was generated via mid LAD ligation. Mid LAD, as opposed to proximal LAD, was chosen to induce mild to moderate myocardial dysfunction while limiting mortality. Fractional shortening, as assessed using echocardiography, revealed myocardial dysfunction in mice after myocardial infarction at 5 and 8 weeks (Figure 3A). Mice treated with S107, a drug that improves cardiac and skeletal muscle function in CHF by reducing RyR-mediated intracellular Ca^{2+} leak,[20,21,35,55] exhibited improved fractional shortening compared with untreated mice after myocardial infarction (Figure 3A). RyR1 Ca^{2+} leak in mouse peripheral, CD-19+, B-lymphocytes was assessed using flow cytometry. B-lymphocytes from CHF mice exhibited a significantly reduced increase in cytosolic Ca^{2+} fluorescence compared with wild-type mice after the addition of the RyR1 agonist, caffeine (Figure 3B; wild type, n=5, 48% ± 6% versus CHF, n=5; 9%±1%; P<0.01). These results are consistent with the presence of chronic RyR1 Ca^{2+} leak and reduced ER Ca^{2+} stores in the CHF lymphocytes. CHF mice treated with S107 exhibited significantly increased ER Ca^{2+} release after the addition of caffeine compared with untreated CHF mice (HF+S107, n=5, 24%±2% versus CHF, n=5; 9% ± 1%; P<0.01). The reduced RyR1 Ca^{2+} leak was associated with reduced 60-day mortality in the S107-treated CHF mice (Figure 3C). These findings support the validity of flow cytometry for specifically assessing RyR1-mediated intracellular Ca^{2+} leak in B-lymphocytes.

To assess whether intracellular Ca^{2+} handling in B-lymphocytes is dysfunctional in humans, patients from 2 independent centers (JMC and CUMC), with and without CHF, as well as CHF patients following LVAD im-

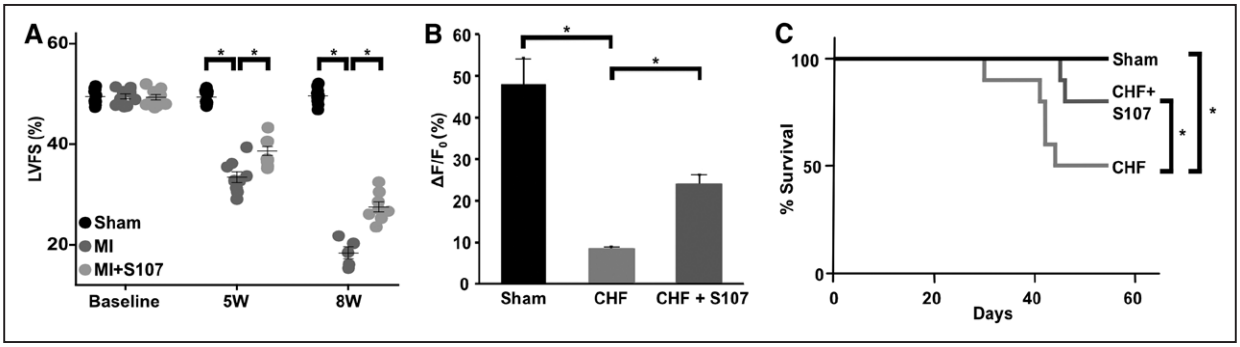

Figure 3. RyR1 Ca^{2+} leak in B-lymphocytes from mice with CHF and improvement with S107 treatment.
A, Left-ventricular fractional shortening (LVFS) at baseline, 5 weeks, and 8 weeks in mice before and after MI, as measured by transthoracic echocardiography. N=10 mice in each group. **B**, Quantification of the % change of mean Fluo-4 fluorescence of CD-19-positive cells in response to 50 mM caffeine. CHF mice exhibit a significantly reduced increase in cytosolic Ca^{2+} in response to caffeine, consistent with the presence of chronic RyR1 Ca^{2+} leak from intracellular ER Ca^{2+} stores. B-lymphocytes from mice treated with S107 exhibit increased cytosolic Ca^{2+} levels after caffeine, consistent with reduced RyR1 Ca^{2+} leak and increased intracellular ER Ca^{2+} stores. Lymphocytes from 5 mice in each group were pooled within their respective groups. Graph represents mean data from 5 caffeine stimulations. **C**, CHF mice treated with S107 in drinking water have decreased mortality compared with untreated mice: Sham (N=10), CHF (N=10), and CHF mice treated with S107 (N=10) were followed for 60 days after MI. Untreated CHF mice exhibited significantly increased mortality at 60 days compared with the Sham group. CHF mice treated with S107 have significantly reduced mortality compared with untreated CHF mice. CHF indicates congestive heart failure; MI, myocardial infarction; and RyR1, type 1 ryanodine receptor. *P<0.05.

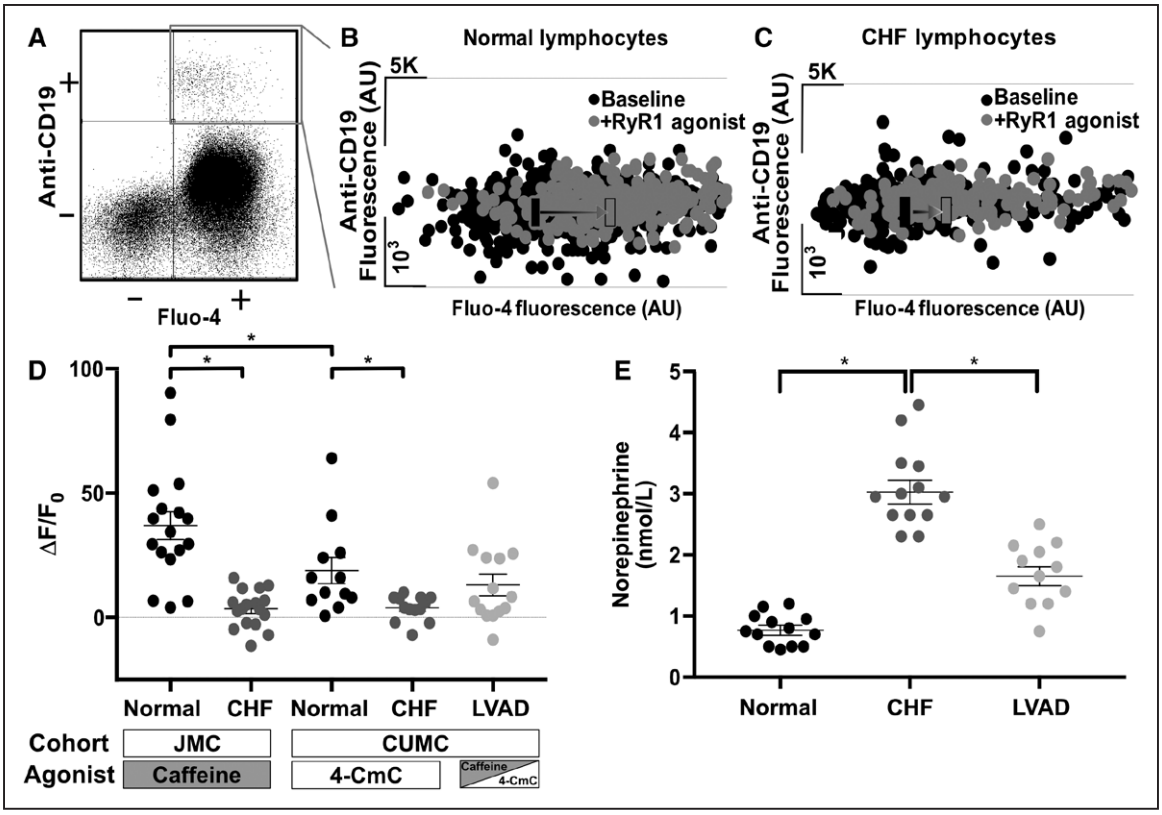

Figure 4. B-lymphocytes exhibit RyR1 Ca^{2+} leak in patients with CHF.
A, Quadrants of flow-cytometry assay. Scatter plot of CD19 fluorescence (B-cell-specific antibody) on the y axis versus Fluo-4 fluorescence (fluorescent cytosolic Ca^{2+} marker) on the x axis. Quadrant of interest (CD-19+ cells, loaded with Fluo4-AM) highlighted in gray. **B** and **C**, Representative graph of B-lymphocyte fluorescence before and after addition of RyR1 agonist (caffeine or 4-CmC) as measured using flow cytometry. Each circle represents a single cell. The cells displayed exhibit both CD19 and Fluo-4 fluorescence. Color bars and direction arrows represent the shift of the mean Fluo-4 fluorescence before and after administration of RyR1 agonist. Data in the normal and CHF examples following the addition of RyR agonist is equally offset to more clearly delineate the before and after data. **D**, Quantification of the % change of mean Fluo-4 fluorescence of CD-19-positive cells at baseline in response to 50 mM caffeine or 0.5 mM 4-CmC. Data segregated by cohort site (JMC and CUMC). JMC normal, N=17, 37% ± 6% versus JMC CHF, N=14, 5% ± 2%, *P*<0.05; CUMC normal, N=13, 17% ± 5% versus CUMC CHF, N=13, 4% ± 1, *P*<0.05; LVAD, N=14, 13% ± 4%). Caffeine induces a greater response in the control population compared with 4-CmC. **E**, Plasma norepinephrine levels measured in normal patients (N=13) and those with CHF (N=13) and LVAD (N=12). Each sample tested in duplicate and average calculated. CHF indicates congestive heart failure; CUMC, Columbia University Medical Center–New York Presbyterian Hospital; JMC, Jacobi Medical Center; LVAD, left-ventricular assist device; and RyR1, type 1 ryanodine receptor. **P*<0.05.

plant, were recruited. Samples obtained from JMC were treated with the RyR agonist caffeine, whereas samples from normal patients and those with CHF recruited at CUMC were treated with the RyR agonist 4-CmC. LVAD samples were obtained from CUMC and treated with either caffeine or 4-CmC. B-lymphocytes from patients with CHF at both centers exhibited a reduced increase in cytosolic Ca^{2+} levels after the administration of an RyR1 agonist compared with patients without CHF (Figures 4A through 4D; JMC normal, n=17, 37%±6% versus JMC CHF, n=14; 5%±2%; *P*<0.05; CUMC normal, n=13, 17%±5% versus CUMC CHF, n=13; 4%±1%; *P*<0.05). These results are consistent with the presence of chronic RyR1-mediated ER Ca^{2+} leak leading to reduced ER Ca^{2+} stores in the CHF cells. We observed that B-lymphocytes from control patients treated with the RyR agonist caffeine had a significantly increased response compared with samples treated with 4-CmC (JMC normal, n=17, 37%±6% versus CUMC normal, n=13; 17%± 5%; *P*<0.05). This finding suggests that caffeine may have a greater effect on ryanodine-releasable stores compared with 4-CmC. B-lymphocytes from the patients with LVAD exhibited a significantly reduced response to caffeine compared with patients from the combined normal cohort. However, there was no significant improvement between the LVAD group and combined CHF groups (Figure 4D; normal, n=30, 28% ± 4% versus CHF, n=27; 4%±1% versus LVAD, n=14, 13%±4%; *P*=N.S.). The LVAD group exhibited relatively larger heterogeneity compared with the CHF group, suggesting that some patients may have experienced improvement (Figure 4D; CHF STDEV=6%, LVAD STDEV=16%). Circulating NE levels were measured in normal patients and those with CHF and LVAD. NE levels were significantly increased in patients with CHF compared with normal controls, and there was a significant reduction observed in the patients with LVAD compared with patients with CHF (Figure 4E); normal, n=13, 0.8 nmol/L±0.07 nmol/L versus CHF, n=13, 3 nmol/L±0.2 nmol/L versus LVAD, n=12, 1.7 nmol/L±0.15 nmol/L; *P*<0.05).

DISCUSSION

Circulating biomarkers that assess cardiac recovery in CHF are needed for monitoring responses to pharmacological and mechanical CHF therapy.[56] Plasma catecholamine levels correlate with CHF severity and initiation of CHF therapy. However, they are difficult and complex to monitor.[8] Persistently elevated circulating NE levels cause pathological RyR2 Ca^{2+} leak in the heart and RyR1 Ca^{2+} leak in skeletal muscle, contributing to CHF progression and risk of sudden death.[13,14,17,21,28,29,36] This study assessed whether elevated circulating NE levels in CHF are associated with pathological RyR1 remodeling and dysfunctional intracellular Ca^{2+} handling/metabolism in circulating B-lymphocytes and whether this improves with LVAD therapy.

Using a flow cytometry-based assay, we observed that RyR1-mediated Ca^{2+} handling in circulating B-lymphocytes is dysfunctional in mice and humans with CHF. The specificity of this assay for measuring RyR1-mediated Ca^{2+} leak was demonstrated by testing 2 separate RyR1 agonists and using CHF mice treated with S107, a drug that specifically targets RyR Ca^{2+} leak.[51] The S107-treated CHF mice exhibited reduced intracellular Ca^{2+} leak and mortality compared with nontreated CHF controls. The association between decreased B-lymphocyte Ca^{2+} leak and mortality in the CHF mice suggests that this assay, once optimized, may have clinical utility for assessing the response to clinically approved CHF therapies and for prognosticating patients with CHF.

We observed that patients with CHF had significantly higher circulating NE levels compared with normal patients, and that these levels correlated with increased RyR1-mediated Ca^{2+} leak in B-lymphocytes. This association supports our hypothesis that chronically elevated circulating NE levels in CHF can cause RyR remodeling and dysfunction in any organ system where the channel is expressed. The specificity of this test for detecting cardiomyopathy, as opposed to comorbid pathologies, which are commonly present in patients with CHF, is supported by the narrow range of responses to RyR1 agonists within the CHF population (–11% to 16%, STDEV 6), suggesting that unknown variables likely play a small role. However, larger studies are needed to adequately address this. Although the patients with LVAD exhibited decreased circulating NE levels because of improved cardiac output, the observed reduction in B-lymphocyte intracellular Ca^{2+} leak was not as robust. This finding is likely because of the heterogeneity of patients with LVAD and raises the possibility that this assay may identify patients with dysfunctional intracellular Ca^{2+} handling despite improvement in circulating catecholamine levels. Future studies should focus on the prognosis of these different patient groups.

We have previously reported in a canine model of CHF that β-blocker therapy reverses PKA phosphorylation of RyR2 and depletion of calstabin2.[28] However, in the present study, despite the fact that >95% of patients with CHF were prescribed β-blocker therapy (Table), the CHF samples tested exhibited PKA phosphorylation of RyR1 and depletion of calstabin1 compared with controls (Figure 1). Possible explanations for this discrepancy include poor compliance with medication and insufficient dosing or length of time on medication in the patients with CHF. Indeed, the lack of differences in the heart rates or blood pressures (systolic, diastolic) between the CHF and control groups (Table) support the hypothesis that these patients with CHF were not physiologically β-blocked.

The contribution of RyR1 Ca^{2+} handling to B-lymphocyte function is poorly understood. Intracellular Ca^{2+} release in B-lymphocytes plays a key role in B-cell activation after antigen challenge.[57] After activation of the antigen receptor complex inositol trisphosphate is produced and binds to inositol trisphosphate receptors on the ER causing Ca^{2+} release from the ER.[58] The ER Ca^{2+} sensors stromal interaction molecule1 and 2 sense the drop in ER Ca^{2+} and activate plasma membrane Ca^{2+} channels Orai1-3, resulting in an influx of extracellular Ca^{2+} into the cell.[58] This process, known as store-operated Ca^{2+} entry, provides the necessary Ca^{2+} to drive gene transcription and the immune response.[57] Ca^{2+} is then pumped back into the ER through SERCA2 and SERCA3.

RyR1 expression in B-lymphocytes has been previously described in studies using a polymerase chain reaction-based restriction fragment-length polymorphism method, [H^3]ryanodine-binding assay, and dose-dependent activation by the RyR-specific activator 4-CmC.[43] [H^3] ryanodine-binding studies have identified a single low-affinity ryanodine-binding site on B-lymphocyte RyR1, in contrast to the high- and low-affinity sites previously reported in skeletal muscle RyR1.[59] It is intriguing that the low-affinity ryanodine-binding site on B-lymphocyte RyR1 behaves similarly to the high-affinity ryanodine-binding site found in skeletal muscle RyR1 (ie, by increasing the Ca^{2+} release response to 4-CmC as opposed to inhibiting it).[43] Although the contribution of RyR1 to B-lymphocyte activation is not well understood, depletion of RyR1-gated stores significantly reduces the Ca^{2+} peak observed after antigen binding to B-cell receptor.[43]

Studies have demonstrated that B-lymphocytes isolated from patients with malignant hyperthermia, a disease characterized by leaky RyR1, exhibit increased sensitivity to caffeine-induced ER Ca^{2+} release, confirming the presence of functional RyR channels in B-lymphocytes.[45] These studies, in addition to our animal data where mice with ischemic cardiomyopathy were treated with S107, a drug that specifically reduces RyR1 Ca^{2+} leak, support the specificity of our assay for assessing RyR1 Ca^{2+} leak in this cell population. The fact that we did not observe any significant differences with our assay between patients with ischemic or nonischemic

cardiomyopathy suggests that the comorbid conditions frequently associated with coronary artery disease were not responsible for our observations. The results of our study also raise the possibility that RyR1 dysfunction in B-lymphocytes may affect immune function in CHF.

The need for monitoring the effectiveness of CHF therapies highlights the importance of tests that can noninvasively assess intracellular Ca^{2+} handling, which is defective in cardiac and skeletal muscle in patients with CHF.[21] In this study, we demonstrate the utility of a flow cytometry-based assay for measuring RyR1 intracellular Ca^{2+} leak in circulating B-lymphocytes as a surrogate for RyR2 function in cardiac and skeletal muscles. By testing the downstream molecular consequences of elevated NE levels, this assay may have superior specificity for CHF and LVAD outcomes compared with available biomarkers such as brain naturetic peptide, whose levels correlate with existing ventricular dysfunction. In fact, we observed a correlation between decreased mortality and reduced B-lymphocyte RyR1-mediated intracellular Ca^{2+} leak in mice.

This B-lymphocyte Ca^{2+} leak assay is relatively simple and inexpensive to perform using standard reagents and equipment available in clinical laboratories. The assay may be useful for following patients serially over time or at a single time point. Larger studies are needed to assess the accuracy and reproducibility of these measurements and to eventually determine whether the degree of RyR1-mediated ER Ca^{2+} leak in B-lymphocytes correlates with CHF interventions and outcomes, and whether this test has prognostic significance in patients with LVAD.

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Correspondence

Andrew R. Marks, MD, Columbia University College of Physicians and Surgeons, Russ Berrie Medical Science Pavilion Room 520, 1150 St Nicholas Avenue, New York, NY 10032; or Richard N. Kitsis, MD, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail richard.kitsis@einstein.yu.edu or arm42@cumc.columbia.edu

Affiliations

Department of Physiology and Cellular Biophysics, Clyde and Helen Wu Center for Molecular Cardiology, Columbia University College of Physicians and Surgeons, New York (A.K., G.S., S.R.R., A.R.M.). Department of Medicine, Division of Cardiology, Columbia University Medical Center, New York (A.K., E.C., S.J.G., D.L.B., P.C.C., M.Y., A.R.M.). Departments of Medicine and Cell Biology, Wilf Family Cardiovascular Research Institute, Albert Einstein Cancer Center, Diabetes Research Center, Albert Einstein College of Medicine, Bronx, NY (R.N.K.). Department of Medicine, Division of Cardiology, Jacobi Medical Center, Bronx, NY (S.I.S.).

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Disclosures

Dr Marks is a consultant and member of the board for a startup company and has shares in ARMGO Pharma Inc, which is targeting RyR2 to prevent heart failure and sudden cardiac death.

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