Heart Failure

Early Remodeling of Perinuclear Ca\textsuperscript{2+} Stores and Nucleoplasmic Ca\textsuperscript{2+} Signaling During the Development of Hypertrophy and Heart Failure

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Background—A hallmark of heart failure is impaired cytoplasmic Ca\textsuperscript{2+} handling of cardiomyocytes. It remains unknown whether specific alterations in nuclear Ca\textsuperscript{2+} handling via altered excitation-transcription coupling contribute to the development and progression of heart failure.

Methods and Results—Using tissue and isolated cardiomyocytes from nonfailing and failing human hearts, as well as mouse and rabbit models of hypertrophy and heart failure, we provide compelling evidence for structural and functional changes of the nuclear envelope and nuclear Ca\textsuperscript{2+} handling in cardiomyocytes as remodeling progresses. Increased nuclear size and less frequent intrusions of the nuclear envelope into the nuclear lumen indicated altered nuclear structure that could have functional consequences. In the (peri)nuclear compartment, there was also reduced expression of Ca\textsuperscript{2+} pumps and ryanodine receptors, increased expression of inositol-1,4,5-trisphosphate receptors, and differential orientation among these Ca\textsuperscript{2+} transporters. These changes were associated with altered nucleoplasmic Ca\textsuperscript{2+} handling in cardiomyocytes from hypertrophied and failing hearts, reflected as increased diastolic Ca\textsuperscript{2+} levels with diminished and prolonged nuclear Ca\textsuperscript{2+} transients and slowed intranuclear Ca\textsuperscript{2+} diffusion. Altered nucleoplasmic Ca\textsuperscript{2+} levels were translated to higher activation of nuclear Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II and nuclear export of histone deacetylases. Importantly, the nuclear Ca\textsuperscript{2+} alterations occurred early during hypertrophy and preceded the cytoplasmic Ca\textsuperscript{2+} changes that are typical of heart failure.

Conclusions—During cardiac remodeling, early changes of cardiomyocyte nuclei cause altered nuclear Ca\textsuperscript{2+} signaling implicated in hypertrophic gene program activation. Normalization of nuclear Ca\textsuperscript{2+} regulation may therefore be a novel therapeutic approach to prevent adverse cardiac remodeling. (Circulation. 2014;130:244-255.)

Key Words: calcium signaling ■ heart failure ■ nuclear envelope ■ remodeling

Heart failure (HF) is characterized by systolic and diastolic dysfunction and abnormalities of intracellular Ca\textsuperscript{2+} handling with disturbed excitation-contraction coupling underlying contractile failure.\textsuperscript{1} Current research focuses on better understanding the mechanisms that lead to disturbed Ca\textsuperscript{2+} handling during progression from cardiac remodeling (such as hypertrophy) to failure.

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The Ca\textsuperscript{2+} cycle in cardiomyocytes that governs contraction and relaxation on a beat-to-beat basis consists of a transient rise in the cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) and subsequent Ca\textsuperscript{2+} decay. Each cytoplasmic [Ca\textsuperscript{2+}] transient (CaT) also elicits a nucleoplasmic CaT.\textsuperscript{2} Much progress has been made toward understanding the role of altered cytoplasmic Ca\textsuperscript{2+} homeostasis in hypertrophy and HF;\textsuperscript{3–5} however, nucleoplasmic [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{nuc}) in HF is understudied and may be critical to cardiac remodeling, because it regulates protein expression through nuclear Ca\textsuperscript{2+}-dependent regulation of gene transcription.\textsuperscript{6,7} Our previous studies indicated that nucleoplasmic CaTs follow distinct kinetics and may be regulated quite differently from cytoplasmic CaTs.\textsuperscript{8} An important aspect of
[Ca\textsuperscript{2+}]\textsubscript{nu} is the nuclear envelope (NE), which not only contributes to nuclear structure and insulation from the surrounding cytoplasm but also controls bidirectional transport of ions (including Ca\textsuperscript{2+}) and macromolecular cargo via nuclear pore complexes (NPCs). The NE is also a functional Ca\textsuperscript{2+} store, akin to the sarcoplasmic reticulum (SR), and contains Ca\textsuperscript{2+} pumps (SR Ca\textsuperscript{2+}-ATPase 2 [SERCA2]) and Ca\textsuperscript{2+} release channels. The regulation of [Ca\textsuperscript{2+}]\textsubscript{nu} via Ca\textsuperscript{2+} release from the NE is also important in Ca\textsuperscript{2+}-mediated gene expression.\textsuperscript{9,10} However, it remains unknown whether specific alterations in NE and nuclear Ca\textsuperscript{2+} handling occur in cardiac disease.

Therefore, we analyzed NE structure and function during cardiac remodeling from hypertrophy to HF in animal models of pressure overload and in nonfailing and failing human hearts. We demonstrate that NE structure, its molecular composition, and nucleoplasmic CaTs undergo significant changes during pressure overload–induced hypertrophy in experimental animal models and in failing human hearts. These nuclear changes precede the changes in cytoplasmic Ca\textsuperscript{2+} dysregulation and thus suggest that altered nucleoplasmic [Ca\textsuperscript{2+}] is an early event during remodeling and may contribute to the development and progression of cardiac hypertrophy and failure via nuclear Ca\textsuperscript{2+}-dependent regulation of gene expression through activation of nuclear Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and nuclear export of histone deacetylases (HDAC). Normalization of impaired [Ca\textsuperscript{2+}]\textsubscript{nu} regulation may therefore represent a novel therapeutic target to prevent adverse cardiac remodeling.

**Methods**

A detailed description of the methods used in the present study can be found in the online-only Data Supplement.

**Animal Models**

All experimental procedures involving animals were approved by the local animal care and use committees according to the Guide for the Care and Use of Laboratory Animals prepared by the US National Academy of Sciences (National Institutes of Health publication No. 85-23, revised 1996). Hypertrophy and HF were induced by transverse aortic constriction (TAC) in C57BL/6 mice or by combined aortic constriction and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) signaling, respectively. \textsuperscript{8} Implantation of permanent aortic banding was performed as described previously.\textsuperscript{7} Hypertrophy and failure via nuclear Ca\textsuperscript{2+}-dependent regulation of gene expression through activation of nuclear Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and nuclear export of histone deacetylases (HDAC). Normalization of impaired [Ca\textsuperscript{2+}]\textsubscript{nu} regulation may therefore represent a novel therapeutic target to prevent adverse cardiac remodeling.

**Human Myocardium**

All procedures involving human myocardium were approved by the ethics committee of the Medical University of Graz (reference No. 20-277 ex08/09) and were performed in accordance with the Declaration of Helsinki. Patient characteristics are summarized in Table I in the online-only Data Supplement.

**Cardiomyocyte Isolation**

Murine, rabbit, and human ventricular cardiomyocytes were isolated by standard enzymatic dissociation procedures.

**Confocal Ca\textsuperscript{2+} Imaging of Nucleoplasmic and Cytoplasmic CaTs**

Simultaneous imaging of nucleoplasmic and cytoplasmic CaTs occurred in cardiomyocytes loaded with Fluo-4 (Molecular Probes, Leiden, The Netherlands) with a confocal imaging system (Zeiss LSM 510 Meta [Carl Zeiss Group, Oberkochen, Germany] or Olympus Fluoview 1000 [Olympus Corp, Tokyo, Japan]) as described previously.\textsuperscript{8} Cardiomyocytes were field-stimulated via 2 platinum electrodes. Isoproterenol (30 nmol/L) and angiotensin II (ATII, 100 nmol/L) were used to investigate the effects of β-adrenergic stimulation and inositol 1,4,5-trisphosphate (IP\textsubscript{3}) signaling, respectively. Cytoplasmic and nucleoplasmic fluorescence signals were transformed into calibrated [Ca\textsuperscript{2+}] by the previously described method.\textsuperscript{9}

**Imaging of Perinuclear Ca\textsuperscript{2+} Stores**

Perinuclear Ca\textsuperscript{2+} stores were visualized in cardiomyocytes loaded with the low-affinity Ca\textsuperscript{2+} indicator mag-fluo-4, AM (Life Technologies, Grand Island, NY), with a confocal imaging system (Zeiss LSM 510 Meta or Olympus Fluoview 1000). The optical slice thickness was ≤0.76 μm. Two-dimensional images were collected at a central depth of the nuclei. The longitudinal axis was drawn through the middle of the nuclei, and tubular structures (longer than 1 μm) were counted along the half of the NE, which contained more invaginations. Rapid application of caffeine (20 nmol/L) occurred in the presence of 20 mM 2,3-butanedione monoxime by wash-in of caffeine for 3 seconds. Caffeine experiments were conducted in cardiomyocytes isolated from C57BL/6 mice that did not undergo any surgery.

**Immunocytochemistry**

Immunocytochemistry was performed as described previously\textsuperscript{11} with the following antibodies: mouse monoclonal anti-NPC protein antibody (ab60080; Abcam, Cambridge, United Kingdom), mouse monoclonal anti-SERCA2a and mouse monoclonal anti-RyR (ryanodine receptor) antibody (MA3-919 and MA3-916; Thermo Scientific, Rockford, IL), goat polyclonal anti-I\textsubscript{P3,R2 antibody (NB100-2466; Novus Biologicals, Littleton, CO), rabbit polyclonal anti-P-CaMKII antibody (ab32678; Abcam, Cambridge, United Kingdom), and rabbit polyclonal anti-HDAC4 antibody (sc-11418; Santa Cruz Biotechnology, Santa Cruz, CA). The specificity of the antibodies was confirmed in Western blots.

**Electron Microscopy and Immunogold Labeling**

Electron microscopic analyses of the NE were performed on human ventricular endocardial trabeculae, prepared as described previously.\textsuperscript{12} For immunogold labeling, ultrathin slices of mouse ventricles were stained with primary antibodies as in Immunocytochemistry, except for the rabbit polyclonal anti-SERCA2a antibody (A010-20; Badrilla Ltd, Leeds, United Kingdom). Goat anti-rabbit IgG (10 nm) and rabbit anti-goat IgG (5 nm) gold conjugates were from British BioCell International (BBI, Cardiff, United Kingdom).

**Fluorescence Resonance Energy Transfer Imaging**

Fluorescence resonance energy transfer (FRET) imaging of CaMKII activation state was performed with the fluorescence resonance energy transfer–based biosensor Camui as described previously.\textsuperscript{13}

**Isolation of Cardiac Nuclei From Human Hearts**

A detailed protocol for isolation of cardiac nuclei from human myocardium can be found in the online-only Data Supplement. The final nuclear fraction (N) was tested for expression of Ca\textsuperscript{2+}-regulating proteins by standard immunoblot techniques with commercially available antibodies (Immunocytochemistry; anti-Nup62 [610497; BD Transduction Laboratories, Oxford, United Kingdom] and anti-Nkx2.5 [sc-14033; Santa Cruz Biotechnology, Santa Cruz, CA]). The anti-Ry-R (1093) antibody used was custom-made.\textsuperscript{14} For quantification, signals were normalized to GAPDH (H) or Porcine staining (H and N).

**Drugs and Solutions**

Unless otherwise indicated, all chemicals were from Sigma-Aldrich (Steinheim, Germany).

**Statistical Analysis**

Data are presented as mean±SEM. Differences between data sets were evaluated with Wilcoxon rank sum test for between-group comparisons and Wilcoxon signed rank test for within-group comparisons.
Correlations were determined with Spearman rank correlation. Significance was accepted at $P<0.05$. Statistical analyses were performed with SPSS version 20 (IBM SPSS, Armonk, NY).

**Results**

**Hypertrophy Versus HF in Mice**

Sham-operated mouse hearts showed identical left ventricular (LV) dimensions and systolic function at either 1 or 6 weeks after surgery (not shown). Assessment of LV end-diastolic diameter and LV end-systolic diameter revealed that 1 week after TAC, mice displayed concentric LV hypertrophy, whereas LV dilation was observed 6 weeks after TAC (Table II and Figure Ia in the online-only Data Supplement). LV systolic function declined progressively, as indicated by ejection fraction reduction of $\approx 8\%$ 1 week and $\approx 50\%$ 6 weeks after TAC (Table II in the online-only Data Supplement). TAC-induced hypertrophy was confirmed by increased heart weight normalized to tibia length or body weight ($n=5–10$; Figure Ib and Ic in the online-only Data Supplement). Six weeks after TAC, mice developed pulmonary edema as manifested by an elevated ratio of lung weight to tibia length (data not shown). These data indicate that pressure overload–induced myocardial remodeling was associated with an early onset of compensatory hypertrophy and subsequent progression to overt HF.

**NE Remodeling in Hypertrophy and HF**

In cardiomyocytes from sham-operated mice and rabbits, loading of perinuclear Ca$^{2+}$ stores with the low-affinity Ca$^{2+}$ indicator mag-fluo-4 revealed an NE and tubular invaginations that traversed the nucleus (Figure 1A, left). Rapid application of caffeine (20 mmol/L) reversibly abolished mag-fluo-4 fluorescence (Figure 1C) both in the NE and in its tubular structures. Fluorescence recovery after depletion was identical in both regions, returning to $\approx 90\%$ of the precaffeine level ($n=7$; Figure 1D). This implies that the NE and its tubular invaginations are functional Ca$^{2+}$ stores capable of releasing and reaccumulating Ca$^{2+}$, both in the nuclear periphery and in the regions within the nucleus otherwise remote from the NE.

A significant increase in the density of tubular invaginations, calculated as the number of invaginations per NE circumference in the central depth of the nucleus, was observed during physiological aging in young (2–5 months) sham-operated mice ($n=90$ nuclei per group; Figure 1B). In contrast, TAC-operated mice exhibited a progressive decrease in NE...
tubular invagination density 1 and 7 weeks after TAC. Similar reductions were observed in cardiomyocytes from nonfailing versus failing rabbit hearts (n=30 nuclei per group) and from nonfailing versus failing human hearts (n=20 nuclei per group), which suggests a misrelationship between the growth of the nuclei and the NE compartment as a general feature of HF, independent of species and pathogenesis of HF. Average nuclear dimensions and number of NE tubular invaginations per nucleus are summarized in Figure II in the online-only Data Supplement.

To confirm the presence of NE invaginations in cardiac tissue (versus isolated myocytes) and to investigate their detailed structure, electron microscopy imaging of nuclei from sections of human ventricular trabeculae was performed. The observed invaginations were lined by the inner and outer nuclear membranes, interrupted by numerous NPCs (Figure 2A, right, red arrows and inset), and filled with cytoplasm (Figure 2A, left and middle).

In summary, these data indicate that the NE of cardiomyocytes contains a network of tubular structures (ie, NE invaginations) that undergoes significant changes during hypertrophy and HF. With the progression of cardiac remodeling, the size of nuclei increases, while at the same time, the number of NE invaginations decreases.

Remodeling of Ca2+-Regulating Proteins in and Around the Nucleus

We also investigated the (peri)nuclear expression of Ca2+ release channels, RyR and IP3 receptor (IP3R), NPCs, and SR Ca2+-ATPase (SERCA), which could have functional consequences for [Ca2+]nuc and HF progression. Immunostaining of cardiomyocytes confirmed the presence of NPCs in both the NE and its invaginations (n=15; Figure 2B, top). In line with mag-fluo-4 stainings (Figure 1A), NPC labeling showed increased nuclear dimensions with reduced number of invaginations in 7-week post-TAC mice and in cardiomyocytes from failing human hearts (quantitative analyses not shown).

In sham-operated mice and in nonfailing human cardiomyocytes, IP3R2 was found in a striated pattern through the cell, in close proximity to the sarcolemma (most prominent) and on the NE. In HF, accumulation of IP3R2 in the perinuclear region was observed (n=15; Figure 2B, middle). Immunogold labeling revealed that IP3R2 was localized more prominently on the inner NE surface (Figure 2D, red arrows), but it was also seen on the outer NE, as well as in the perinuclear region (Figure 2D, bottom).

A punctate pattern with a striated organization was observed for RyR2, and combined with nuclei staining, it was revealed (Figure III in the online-only Data Supplement) that RyR2 did not penetrate into the nuclei but rather formed a “cage” around them, as described previously. A large reduction of RyR2 staining in perinuclear regions, in particular in the longitudinal direction, was observed in 7-week post-TAC mice and in cardiomyocytes from failing human hearts (n=15; Figure 2B, middle; Figure III in the online-only Data Supplement). Immunogold labeling confirmed that RyR2 was not expressed on but only near the NE (Figure 2C, top and middle). As expected, RyR2 was seen in SR-like structures surrounding T-tubules (Figure 2C, bottom).

SERCA2a expression exhibited a characteristic network-like pattern that reflected the SR throughout the cytoplasm. In addition, SERCA2a staining was strongly positive on the NE and its invaginations in sham mouse and nonfailing human cardiomyocytes. Consistent with our earlier observation of blunted invagination density in HF, such structures were absent with SERCA2a immunostaining in either murine or human failing myocytes (n=15; Figure 2B, bottom). Immunogold labeling indicated that SERCA2a was expressed primarily on the outer nuclear membrane (n=11; Figure 2E; Figure IV in the online-only Data Supplement) and that its expression on the NE was decreased significantly in 7-week post-TAC mice (n=6; Figure 3C, bottom right).

Altered expression levels of Ca2+-regulating proteins were confirmed by Western Blot analysis of isolated human cardiac nuclei (Figure V in the online-only Data Supplement). Nuclear fractions from failing human myocardium exhibited a significant decrease in SERCA2a and RyR2 expression (which was detectable most likely because of remnants of perinuclear regions sticking to the nuclei), whereas IP3R2 levels were increased compared with nonfailing hearts.

[Ca2+]nuc and [Ca2+]cyto During Electric Stimulation

Fluo-4 fluorescence signals recorded during electrically stimulated CaTs (Figure 3A) were transformed into [Ca2+]nuc and [Ca2+]cyto by our compartment-specific calibration methods (Figure 3B). No differences in CaTs were observed between the 2 groups (1 and 7 weeks) of sham-operated mice (data not shown). One week after TAC, diastolic [Ca2+]nuc and [Ca2+]cyto were increased significantly, and they increased further 7 weeks after TAC (Figure 3C). The increase of diastolic [Ca2+]nuc was much more pronounced than that of [Ca2+]cyto (Figure VI in the online-only Data Supplement). Peak systolic [Ca2+] remained unchanged in either compartment (data not shown). Early in hypertrophy, CaTs were slowed and CaT amplitudes were reduced only in the nucleoplasm. At 7 weeks after TAC, similar changes of CaTs also occurred in the cytoplasm (Figure 3D and 3E).

The NE invaginations and SERCA2/RyR2 distribution suggest the following working model of nuclear CaTs. The rise in [Ca2+]nuc is driven mainly by an RyR2-dependent rise in perinuclear [Ca2+]nuc, and the primarily cytosolic facing SERCA2 distribution suggests that Ca2+ may largely have to diffuse out of the nucleus to be pumped back into the NE and SR. These aspects would slow the rise and fall of nuclear versus cytosolic CaTs (as observed) and could be exacerbated by the reduction in invaginations during the progression to HF. We measured this diffusional delay during development of HF in the TAC mice in the present study by measuring CaTs at the surface and center of both the nucleus and cytosol (Figure 3F through 3J; Figure VII in the online-only Data Supplement). Kinetics of cytosolic CaTs were spatially uniform and unaltered 1 week after TAC (n=10; Figure VII in the online-only Data Supplement). However, at 7 weeks after TAC, there was some slowing of the central CaT versus the subsarcomemmal CaT (Figure VII in the online-only Data Supplement), presumably caused by the reported reduction in T-tubular density that occurs in HF. In contrast, nuclear CaT propagation velocity (difference in time to peak between subnucleolemmal
Figure 2. Nuclear envelope (NE) invaginations and localization of Ca\(^{2+}\)-regulating proteins in nonfailing and failing cardiomyocytes. A, Transmission electron micrograph of a nucleus from human left ventricular trabeculae (left), zoomed in on the NE invaginations (middle and right) and nuclear pore complexes (NPC; red arrows and inset). B, Original 2-dimensional images of cardiomyocytes isolated from sham-operated mice and mice subjected to transverse aortic constriction (TAC) 7 weeks after the intervention (left) and nonfailing and failing human cardiomyocytes (right) after immunostaining for NPC (top), inositol 1,4,5-trisphosphate receptor (IP\(_{3}\)R; middle), ryanodine receptor (RyR; middle) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a; bottom). Scale bars, 5 μm. Transmission electron micrograph of control mouse myocardium stained for (C) RyR, NE and its surroundings (top and middle), and T-tubules (TT) and their surroundings (bottom); (D) IP\(_{3}\)R, zoomed in on NE (top) and perinuclear region (bottom); and (E) SERCA2a, zoomed in on NE (top and middle), distribution between inner and outer nuclear membrane (n=11; bottom, left), and expression level on NE in sham- vs TAC-operated mouse myocardium (n=6; bottom, right). *P<0.05 vs inner nuclear membrane or 7-week post–sham operation mice. C through E, Red arrows and insets indicate gold particles. Scale bars, 0.2 μm.
and central nucleoplasmic region divided by the distance) was already slowed significantly at 1 week after TAC (Figure 3J, right). Selective slowing of the central nucleoplasmic CaTs 1 week after TAC (Figure 3I through 3J) also led to a more pronounced increase in diastolic central [Ca²⁺]nuc as soon as 1 week after TAC (Figure 3H). This observation might be particularly relevant at high stimulation frequencies, when diastolic [Ca²⁺]nuc is more affected than [Ca²⁺]cyto because of its slower kinetics. In the rabbit HF model, we also observed elevated diastolic [Ca²⁺]nuc and slowed kinetics of [Ca²⁺]nuc rise (Figure VIII in the online-only Data Supplement).

Importantly, in human myocytes (both for moderate and severe HF), we saw quite similar effects as in the 1- and 7-week post-TAC mice (Figure 4). Although a low number of moderately failing hearts is a limitation of the study, the present results suggest that early alterations in CaTs that occur selectively in the nucleus might be clinically relevant for the progression of cardiac remodeling in HF patients.

**Correlation of NE Invaginations With Nuclear CaT Kinetics**

In mouse and rabbit cardiomyocytes, we simultaneously quantified NE invaginations (with mag-fluo-4) and spatiotemporal aspects of cytoplasmic and nucleoplasmic CaTs (with rhod-2). Figure 5A shows that nuclear CaT propagation was slowed in cardiomyocytes with lower NE invagination density. Correlation analysis indicated a strong inverse correlation between the density of NE invaginations and Ca²⁺ propagation to and from the nucleus (time from peak [Ca²⁺] to 50% decline [DTₜ₀]) in both sham and HF conditions (Figure 5B).

The present results suggest that early alterations in CaTs that occur selectively in the nucleus might be clinically relevant for the progression of cardiac remodeling in HF patients.
Frequency-Dependent Changes of Nucleoplasmic CaTs

Figure 6A and 6B shows recordings in which stimulation frequency was gradually increased from 0.5 to 5 Hz (mouse cells) or from 0.2 to 1 Hz (rabbit cells). In all 3 groups of mice, diastolic [Ca\textsuperscript{2+}] in the nucleus and cytoplasm rose with an increase in stimulation frequency (Table III in the online-only Data Supplement). However, during early remodeling (TAC 1 week), diastolic [Ca\textsuperscript{2+}]\textsubscript{nuc} was already elevated at a very low stimulation rate (in contrast to diastolic [Ca\textsuperscript{2+}]\textsubscript{cyto}). At faster stimulation rates, diastolic [Ca\textsuperscript{2+}]\textsubscript{nuc} also rose much more prominently than [Ca\textsuperscript{2+}]\textsubscript{cyto}. In failing cardiomyocytes (TAC 7 weeks), these changes in nucleoplasmic and cytoplasmic diastolic [Ca\textsuperscript{2+}] were exacerbated. Systolic peak [Ca\textsuperscript{2+}] at 1 and 7 weeks after TAC was comparable to the sham group in both compartments at most frequencies. The increase in diastolic [Ca\textsuperscript{2+}] resulted in a progressive decline in CaT amplitude with faster stimulation rates. The reduction of CaT amplitude started at a lower frequency in the nucleoplasmic versus cytoplasmic compartment. Similar changes were observed in cardiomyocytes from nonfailing versus failing rabbit hearts (Figure 6B).

In further experiments, we investigated whether β-adrenergic stimulation could diminish or prevent the disproportionate rise in [Ca\textsuperscript{2+}]\textsubscript{nuc} in hypertrophied and failing cardiomyocytes to increased stimulation frequencies (Figure 6A, right). β-Adrenergic stimulation greatly accelerates CaT decay and thereby might blunt the frequency-dependent increases in diastolic [Ca\textsuperscript{2+}].\textsuperscript{17} Application of isoprenaline (30 nmol/L) resulted in a robust increase of both nucleoplasmic and cytoplasmic CaTs in control cardiomyocytes; however, the preferential rise in diastolic [Ca\textsuperscript{2+}]\textsubscript{nuc} was still observed and again was more pronounced as HF progressed (1 versus 7 weeks after TAC). Average data and statistical analysis related to Figure 6A are summarized in Table III in the online-only Data Supplement.

Collectively, these data revealed 3 important points: (1) The frequency-dependent increase in diastolic [Ca\textsuperscript{2+}]\textsubscript{nuc} was much larger than the increase in diastolic [Ca\textsuperscript{2+}]\textsubscript{cyto}; (2) it persisted with β-adrenergic stimulation; and (3) this effect was even more pronounced in failing myocytes.

Frequency-Dependent Activation of Nuclear CaMKII and Nuclear Export of HDAC

In cardiomyocytes from sham-operated mice stimulated at low frequency (0.5 Hz), there was only weak staining of the autonomously active form of CaMKII, phospho-CaMKII, in the cytoplasm and within the nucleus (Figure 6C). A higher stimulation frequency (5 Hz) increased CaMKII phosphorylation, corresponding to the increase in [Ca\textsuperscript{2+}]\textsubscript{nuc} and [Ca\textsuperscript{2+}]\textsubscript{cyto}.
under the same pacing conditions, with much larger increases in the nucleus (particularly in the NE), especially and markedly in HF (n=25; Figure 6C). The phospho-CaMKII signals were prevented by preincubation with KN-93 (1 μmol/L), which confirmed the functional basis of the observed signals.

We also expressed the fluorescence resonance energy transfer–based biosensor CaMKII activity reporter Camui in control and HF rabbit cardiomyocytes. Camui displayed similar cellular distribution as the endogenous phospho-CaMKII detected by immunocytochemistry, consistent with known CaMKII localization. Camui signals indicated significantly higher CaMKII activation in the nucleus and cytoplasm of failing cardiomyocytes at rest (n≥32; Figure 6D).

HDAC4 is a transcriptional regulator that is a downstream effector of CaMKII in the nucleus. That is, CaMKII binds to and phosphorylates HDAC4 to drive HDAC4 nuclear export and derepression of hypertrophic transcription. We found that in HF versus control myocytes, HDAC4 was less nuclear at low frequency stimulation and was more readily driven out by increasing the pacing frequency (n≥20; Figure 6E), as expected given the frequency-dependent increase in nuclear CaTs and CaMKII activation (Figure 6A through 6C).

Role of IP₃ in Regulation of Nucleoplasmic CaTs
IP₃ is an important regulator of nucleoplasmic CaTs. Inhibition of IP₃Rs (with 2-aminoethoxydiphenyl borate) causes selective decreases in diastolic nucleoplasmic [Ca²⁺] in cardiomyocytes. Furthermore, perinuclear IP₃ expression is augmented in HF (Figure 2; Figure IV in the online-only Data Supplement).

Figure 7A shows line-scan images of CaTs from electrically stimulated cardiomyocytes in the absence or presence of ATII, which is known to cause IP₃ production in cardiomyocytes. In sham-operated mice, application of ATII increased diastolic [Ca²⁺] in both the cytoplasm and nucleus (n=15; Figure 7B and 7C). The increase in [Ca²⁺]nuc was much larger than the increase in diastolic [Ca²⁺]cyto, and this effect was augmented in 7-week post-TAC cardiomyocytes. Large increases in diastolic [Ca²⁺] led to significant increases in systolic [Ca²⁺] in both compartments (Figure 7D), whereas CaT amplitude remained unaltered. ATII raised systolic [Ca²⁺] by similar levels in sham versus TAC cardiomyocytes, whereas on ATII application, systolic [Ca²⁺] was significantly higher in 7-week post-TAC cardiomyocytes than in controls. Application of 2-aminoethoxydiphenyl borate (3 μmol/L) completely blocked the effect of ATII (data not shown), consistent with the ATII effects being IP₃ mediated.

Discussion
The present study is the first to provide direct, compelling evidence for structural and functional changes of the NE and nucleoplasmic Ca²⁺ handling during cardiac remodeling and HF in mouse and rabbit models of pressure overload and in human hearts. The progressive decrease in NE invagination density and changes in the Ca²⁺-regulatory protein expression patterns (schematically summarized in Figure 8) were associated with alterations of nucleoplasmic [Ca²⁺] handling and consequent activation of gene transcription via the nuclear CaMKII-HDAC4 axis in electrically stimulated hearts.
Figure 6. Frequency-dependent changes of nucleoplasmic (nuc) vs cytoplasmic (cyto) \([\text{Ca}^{2+}]\) transients (CaTs), phosphorylation of \(\text{Ca}^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), and histone deacetylase 4 (HDAC4) nuclear export in nonfailing and failing cardiomyocytes. A, Original recordings of nucleoplasmic (red) vs cytoplasmic (black) CaTs of cardiomyocytes from sham-operated mice (top) and mice subjected to transverse aortic constriction (TAC; 1 [middle] and 7 [bottom] weeks after intervention) during increases of stimulation frequency from 0.5 to 5 Hz. B, Original recordings of nucleoplasmic (red) vs cytoplasmic (black) CaTs of cardiomyocytes from nonfailing (top) and failing rabbits (bottom) during increases of stimulation frequency from 0.2 to 1 Hz. C, Original confocal images (top) of cardiomyocytes from sham- and TAC-operated mice (1 and 7 weeks after intervention) immunostained for phospho-CaMKII (P-CaMKII) after 10 minutes of stimulation at 0.5 (left) or 5 (right) Hz. Average P-CaMKII fluorescence values (bottom left) and increases in P-CaMKII levels were calculated as difference from sham (\(\Delta F\), bottom right) from 25 cardiomyocytes. *\(P<0.05\) vs 0.5 Hz (left) or 1 week post-TAC group (right); #\(P<0.05\) vs cytoplasm. D, Original confocal images of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) emission signals from nonfailing and failing rabbit cardiomyocytes expressing Camui (left) and average data of Camui activation (\(F_{\text{CFP}}/F_{\text{YFP}}\)) in nucleus and cytoplasm of 32 nonfailing and 90 failing cardiomyocytes at baseline conditions (right). *\(P<0.05\) vs nonfailing. E, Original confocal images (left) of nonfailing rabbit cardiomyocytes immunostained for HDAC4 after 15 minutes’ stimulation at 0.2 (top) or 1.5 (bottom) Hz and average values of nucleoplasmic/cytoplasmic HDAC4 fluorescence ratio in 28 nonfailing and 20 failing cardiomyocytes. *\(P<0.05\) vs 0.2 Hz nonfailing; #\(P<0.05\) vs 0.2 Hz failing. C through E, Scale bars=20 \(\mu\)m.
cardiomyocytes isolated from hypertrophied and failing hearts. Changes in nuclear CaTs occurred before cytoplasmic CaTs were affected, with an onset so early that they may well have been involved in the development and progression of hypertrophy and HF.

The NE of numerous cell types (including cardiomyocytes) contains invaginations that include deep, branching tubular structures (for review, see Malhas et al). As caffeine experiments (Figure 1C) demonstrated, the NE and its invaginations represent functional Ca2+ stores capable of releasing and reaccumulating Ca2+. It is tempting to speculate that similar to T-tubular sarcolemmal invaginations, which are critical for coordinated Ca2+ cycling throughout the myocyte, nuclear tubular invaginations may be critical for minute control of nucleoplasmic Ca2+ release and removal. Recent work suggested that the NE invaginations might be an artifact caused by NE folding attributable to the shorter sarcomere length in isolated cells. However, we also observed NE invaginations in multicellular cardiac tissue (Figure 2A). Furthermore, the fact that sarcomeres in cardiomyocytes rhythmically shorten and lengthen during the contraction-relaxation cycle suggests that NE invaginations might change in number and depth in the beating heart.

There are several important consequences of the NE invaginations penetrating into the nucleus. In general, they facilitate intranuclear regulation of ions and transcription factors that travel between cytoplasm and nucleoplasm by decreasing the diffusion delay and by increasing membrane surface area, which may be critical for the regulation of gene transcription. The presence of NPCs on the NE invaginations ensures effective nucleoplasmic-sarcolemmal ion diffusion and cargo transport in regions that would otherwise be remote from the nuclear periphery. Expression of SERCA pumps on the invaginations enables Ca2+ removal from deep within the nucleus, but because most SERCA2 pumps face the cytoplasmic side, their position with respect to nuclear pores may be important in shaping nucleoplasmic CaT kinetics.

Our working hypothesis, based on NE invaginations and NPC, SERCA2, and RyR2 distribution is the following (Figure 8): Normally, the rise in [Ca2+]nuc is driven mainly by the RyR2-dependent rise in [Ca2+]cyto that occurs during excitation-contraction coupling, and Ca2+ diffusion via NPCs (including invaginations) is the cause of slower rise times and peak [Ca2+]nuc. NE SERCA2 mainly faces the cytoplasm (where there is also much more SERCA on SR), which indicates that most Ca2+ may have to diffuse out of the nucleus to be pumped back into the NE and SR (which would account for the much slower [Ca2+]nuc decline). The loss of NE invaginations which occurs early in HF (in mouse, rabbit, and human) would reduce NPC and SERCA within the nuclear core, exacerbate the slowing of nuclear CaTs, and elevate diastolic [Ca2+]nuc, especially at higher heart rates. Indeed, we directly measured all those effects here, even in the presence of β-adrenergic stimulation. The overall decrease in SERCA functional expression in HF slowed [Ca2+]cyto decline, which further increased diastolic [Ca2+]nuc, and consequent higher CaMKII activation level that we observed in myocytes from the early TAC group at higher pacing frequencies may be causally involved in the remodeling processes that lead to HF, in particular when one considers the often elevated heart rates of patients with HF. Indeed, the SHIFT (Systolic Heart Failure Treatment With the I Inhibitor Ivabradine) trial data demonstrated a beneficial effect of lowering the heart rate by ivabradine in patients with advanced systolic heart failure, both in terms of attenuated LV remodeling and less frequent hospitalizations.

Inositol 1,4,5-trisphosphate–dependent changes of nucleoplasmic vs cytoplasmic [Ca2+] transients (CaTs) in nonfailing and failing mouse cardiomyocytes. A, Line-scan images of cytoplasmic and nucleoplasmic CaTs in a nonfailing (top) and failing (bottom) mouse cardiomyocyte without (normal Tyrode, NT) or with (+ ATII) angiotensin II (100 nmol/L). B, Averaged original recordings of nucleoplasmic (red) vs cytoplasmic (black) CaTs of cardiomyocytes from sham-operated mice (top) and mice subjected to transverse aortic constriction (TAC; bottom) in the absence (left) or presence (right) of ATII. Average diastolic (C) and systolic (D) [Ca2+] (n=15 cardiomyocytes per group). *P<0.05 vs NT; #P<0.05 vs sham ATII. Dia indicates diastolic; and Sys, systolic.
The simple working hypothesis above becomes complicated somewhat by the IP\(_R\) localization that was preferentially (but not exclusively) facing the nucleoplasm. G\(_{\alpha}\)-coupled receptors (\(\alpha\)-adrenergic, endothelin, and ATII receptors) that induce IP\(_R\) production may be more activated during HF progression.\(^{22,23}\) However, nuclear calcineurin is increased in human HF\(^{20}\) and required for full transcriptional effects of NFAT.\(^{30}\) Similarly, in adult ventricular myocytes, \(\alpha\)-adrenergic receptor activation selectively increased nuclear CaMKIIδ phosphorylation (without altering SR-associated CaMKII), an effect attributed to Ca\(^{2+}\) mobilized through nuclear IP\(_R\)-sensitive stores.\(^{31}\) Accumulation of IP\(_R\)-s in this region in cardiomycocytes from failing hearts would support the idea of enhanced NFAT and CaMKII signaling and activation of a hypertrophic gene program.

During the past 2 decades, great advances have been made in understanding alterations in cytoplasmic ion homeostasis in cardiomycocytes during the development of hypertrophy and HF. Here, we show for the first time how nucleoplasmic Ca\(^{2+}\) homeostasis is altered during this hypertrophy-HF process. We provide evidence for structural and functional alterations of the nucleus and nuclear Ca\(^{2+}\) signaling as remodeling progresses. Importantly, the changes were observed not only in animal models of hypertrophy and HF but also in human HF. The present results implicate [Ca\(^{2+}\)]\(_{\text{nuc}}\) as an important determinant of cardiac remodeling that may contribute to the development and progression of hypertrophy and HF. Normalization of nucleoplasmic Ca\(^{2+}\) regulation may therefore be a novel therapeutic approach to prevent adverse cardiac remodeling.

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We thank Anthony Lai for providing the RyR antibody, Eva-Maria Gutsch and Elisabeth Bock for excellent technical assistance, and Kenneth Ginsburg for help with rabbit HF model.

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Disclosures
None.

References

CLINICAL PERSPECTIVE

Hypertrophy and heart failure are severe and widespread cardiac diseases associated with increased morbidity and mortality. Understanding the underlying cellular mechanisms is crucial for developing new treatment strategies and thereby improving prognostic. Numerous studies have identified impaired cytoplasmic Ca2+ handling of cardiomyocytes as underlying contractile failure in hypertrophy and heart failure. Here, we show for the first time how nucleoplasmic Ca2+ homeostasis is altered during this hypertrophy–heart failure transition. We provide compelling evidence that nuclear envelope structure, its molecular composition, and nucleoplasmic Ca2+ transients undergo significant changes as remodeling progresses. These nuclear changes precede the changes in cytoplasmic Ca2+ dysregulation, thus suggesting that altered nucleoplasmic [Ca2+] is an early event during remodeling and may contribute to the development and progression of cardiac hypertrophy and heart failure. Importantly, the changes were observed not only in animal models of hypertrophy and HF but also in nonfailing, moderately failing, and end-stage failing human hearts, hence strengthening their general applicability and clinical relevance. In conclusion, the present work presents a new paradigm for cardiac hypertrophy and failure, in which early remodeling of nuclear calcium homeostasis is an important factor for the development and progression of this common and severe cardiac disease. Normalization of impaired nucleoplasmic Ca2+ regulation may therefore be a novel therapeutic approach for the prevention of adverse cardiac remodeling.
Early Remodeling of Perinuclear Ca\textsuperscript{2+} Stores and Nucleoplasmic Ca\textsuperscript{2+} Signaling During the Development of Hypertrophy and Heart Failure

Senka Ljubojevic, Snjezana Radulovic, Gerd Leitinger, Simon Sedej, Michael Sacherer, Michael Holzer, Claudia Winkler, Elisabeth Pritz, Tobias Mittler, Albrecht Schmidt, Michael Sereinigg, Paulina Wakula, Spyros Zissimopoulos, Egbert Bisping, Heiner Post, Gunther Marsche, Julie Bossuyt, Donald M. Bers, Jens Köckskämper and Burkert Pieske

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Supplemental Material

Materials and Methods

Cell Preparation

Murine myocyte isolation

The procedure was approved by the local Animal Care and Use Committees according to the Guide for the Care and Use of Laboratory Animals prepared by the U.S. National Academy of Sciences (National Institutes of Health publication No. 85-23, revised 1996). Adult ventricular myocytes were isolated by a liberase-based Langendorff perfusion protocol as described in detail previously. All in vitro experiments were conducted at room temperature (22-24°C).

Human myocyte isolation

The procedure was approved by the Ethical Committee of the Medical University of Graz (ref. No. 20-277 ex08/09) and was carried out in accordance with the Declaration of Helsinki. Myocardial samples were obtained from the explanted failing hearts at the time of transplantation (N=5) and from unused donor hearts (N=8). Heart function was evaluated by transthoracic echocardiography and divided into three groups: non-failing (EF≥55%), moderately failing (55%>EF>35%) and severely failing (EF≤35%). In severely failing group, only patients with dilated cardiomyopathy (DCM) were included in this study. Patient characteristics are summarized in Table S1.

The procedure for cell isolation was modified after Sipido et al. Immediately following the resection, hearts were perfused with Custodiol® cardioplegic solution in the operating room at the time of surgery. Hearts were kept at 4°C in Custodiol® solution supplemented with 2,3-butanedione-monoxyime (BDM). A wedge of the left ventricular wall with its perfusing coronary
artery was carefully excised, the artery was cannulated and perfused with Tyrode’s solution (in mM: 130 NaCl, 5.4 KCl, 1.2 MgSO₄, 1.8 CaCl₂, 20 HEPES, 10 taurine, 12.5 glucose, 2 pyruvate and 1 Na-L-lactate; pH 7.2 with NaOH). After cannulation the tissue wedge was perfused at 37°C with a Ca²⁺-free Tyrode’s solution for 10 min followed by enzyme perfusion for 25 min (liberase 0.15 mg/ml, trypsin 0.056 mg/ml, in 0.05 mM CaCl₂ Tyrode’s solution). The tissue was sectioned in slices of 5-7 mm and minced in 0.1 mM CaCl₂ Tyrode’s solution. The preparation was filtered and a small part of the cell yield was fixed for immunocytochemistry. In the remainder the solution was stepwise replaced by Tyrode’s solution containing 1.8 mM CaCl₂. Ca²⁺-tolerant cell yield was 5-20% and it was used for CaT recordings.

**Rabbit myocyte isolation**

Isolation protocol was performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of California, Davis Institutional Animal Care and Use Committee. Adult rabbit ventricular cardiomyocytes were isolated from New Zealand white rabbits by standard enzymatic dissociation as described previously.

**Animal disease models**

**Mouse hypertrophy and heart failure model**

The surgical procedure was approved by the responsible government agency (ref. No. BMWF-66.010/0062-II/10b/2010).

Eight to eleven weeks old C57BL/6 wild-type mice of 21±1 g (females) and 24±1 g (males) body weight underwent transverse aortic constriction (TAC) surgery as described previously. An age- and weight-matched group of mice underwent sham procedure consisting of aortic exposure
without ligation and served as control. Ventricular cardiomyocytes were isolated 1 and 7 weeks after TAC/sham.

One and six weeks after sham/TAC intervention, transthoracic echocardiography was performed and left ventricular fractional shortening (FS) and left ventricular mass (LV\textsubscript{mass}) was calculated as previously described.

**Rabbit heart failure model**

HF was induced in New Zealand White rabbits by combined aortic insufficiency and stenosis as previously described. HF progression was assessed by 2D echocardiography and rabbits were studied ~12 months later, when end-systolic dimension exceeded 12 mm. All protocols followed the Guide for the Care and Use of Laboratory Animals and were approved by the University of California, Davis Institutional Animal Care and Use Committee.

**Confocal Ca\textsuperscript{2+} imaging of nucleoplasmic and cytoplasmic CaTs**

Cells were loaded with the Ca\textsuperscript{2+}-sensitive fluorescent dye Fluo-4 (Molecular Probes, Leiden, The Netherlands) as described previously and placed on the stage of an inverted microscope equipped with a Plan Neofluar 40x/1.3 N.A. oil-immersion objective and a Zeiss LSM 510 Meta confocal laser point scanning system (Zeiss, Jena, Germany). Alternatively, an Olympus Fluoview 1000 confocal microscope (Olympus, Center Valley, PA, USA) was used. Excitation and emission wavelengths were 488 nm and >515 nm, respectively. The pinhole was set to 1 Airy unit, resulting in an optical slice thickness of 0.9 μm. The confocal plane was set to the middle (z-axis) of the nuclei, ensuring that only fluorescence originating from the nucleoplasm was collected. A 512 pixel scan line was positioned to include the nucleus, and scanned every 1.27 ms. Consecutive scan lines were stacked over time and visualized as 2D image.
For quantification of nucleoplasmic vs. cytoplasmic CaTs cells were field-stimulated via two platinum electrodes at 1 Hz (mouse) or 0.5 Hz (rabbit and human). To measure the frequency-dependent changes in CaTs, stimulation frequency was gradually increased from 0.5 Hz to 5 Hz (mouse) or from 0.2 Hz to 1.5 Hz (rabbit). Isoprenaline (30 nM) and angiotensin II (ATII, 100 nM) were used to investigate the effects of β-adrenergic stimulation and IP₃ signaling, respectively. Cytoplasmic and nucleoplasmic fluorescent signals were transformed into calibrated [Ca²⁺] using the previously described method ⁶.

**Isolation of cardiac nuclei from human hearts**

Ventricular myocardium from human hearts (N=12) was homogenized (H), filtered, diluted with water (3:1), and layered onto 1.5 volumes of buffer A (in mM: Hepes (pH 7.2) 10; sucrose 320; MgCl₂ 3; disodium pyrophosphate 25; EGTA 5; PMSF 1; leupeptin 0.001; pepstatin 0.001; DTT 1). Following a first centrifugation at 700g for 10 min at 4°C, the pellet (P₁) containing the nuclei was suspended in hypotonic buffer A1 (buffer A without disodium pyrophosphate and with only 2.4 mM sucrose). The suspension was centrifuged at 50.000 g for 90 min at 4°C. Afterwards, the pellet (P₂) containing the nuclei was suspended in nucleus storage buffer (in mM: Hepes (pH 7.6) 50; sucrose 320; magnesium acetate 5; Na₃EDTA 0.1; EGTA 1; PMSF 0.1; leupeptin 0.001; pepstatin 0.001; DTT 1). Finally, this suspension was spun down at 800g for 10 min, and the final nuclear pellet (N) was suspended in 0.1 ml nucleus storage buffer. Immunoblot analysis of the various fractions (H = whole tissue homogenate; S₁ and S₂ = supernatant after first and second centrifugation step, respectively; P₁ and P₂ = pellet after first and second centrifugation step, respectively; N = final purified nuclear fraction) revealed enrichment of nuclear markers (Nup62, Nkx2.5) in P₁, P₂, and N, and the absence of the cytoplasmic marker GAPDH in these fractions.
(Figure S4a). In addition, microscopic analysis showed intact isolated nuclei in the final nuclear fraction (Figure S4b).

The final nuclear fraction (N) was tested for expression of Ca\(^{2+}\)-regulating proteins (Figure S4c) using standard immunoblot techniques with commercially available antibodies (see section Immunocytochemistry; anti-Nup62 (610497, BD Transduction Laboratories, Oxford, UK) and anti-Nkx2.5 (sc-14033, Santa Cruz Biotechnology, Santa Cruz, CA, USA)). The anti-RyR (1093) antibody used was custom-made \(^7\). For quantification, signals were normalized to GAPDH (H) or Ponceau staining (H and N).

**Electron microscopy and immunogold labeling**

Electron microscopic analyses of the NE were performed on human ventricular endocardial trabeculae, prepared as previously described \(^8\). The samples were fixed at 4°C over night in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde and 2.5% formaldehyde. The samples were post-fixed for 2h at room temperature in the same buffer additionally containing 2% osmium tetroxide, rinsed and dehydrated in a series of graded ethanol solutions. Using propylene oxide as an intermedium, the samples were embedded in TAAB epoxy resin (TAAB, Aldermaston, UK). 60 nm thick sections were cut on a Leica UCT ultramicrotome (Leica Mikrosysteme Handelsges.m.b.H., Vienna, Austria). The sections were stained in lead citrate and uranyl acetate using a Leica EM AC20 contrasting instrument, and then visualized using a FEI Tecnai G²20 transmission electron microscope (FEI, Eindhoven, Netherlands), as described previously \(^9\).

For immunogold labeling, mouse hearts were quickly excised and perfused with PBS. Ventricles were placed in fixing solution (0.1% glutaraldehyde, 2% paraformaldehyde, in 0.1 % sodium-phosphate buffer, pH 7.4) and sliced with a razor blade in cubes sized 1-2 mm\(^3\). After 1-3h, tissue
pieces were rinsed and dehydrated in a series of graded ethanol solutions. Samples were incubated in a mixture of LR White Embedding Medium and 80% ethanol (1:1) for 1h and twice in pure LR White for 1h. Resin was allowed to polymerize for 24h at 50ºC. Ultrathin sections were cut and mounted on nickel grids. Grids were washed in phosphate buffered gelatine (PBG) solution (0.8% BSA in PBS, 0.1% fish gelatine, 0.1M glycin) and incubated in blocking solution (5% normal goat (for goat anti-rabbit IgG gold conjugate) or normal rabbit (for rabbit anti-goat IgG gold conjugate) serum in PBG solution containing 0.05% Tween20). Grids were incubated for 2h with primary anti-IP_{3}R and anti-RyR antibodies as mentioned in Immunocytochemistry section, and a rabbit polyclonal anti-SERCA2a antibody (A010-20, Badrilla, Leeds, UK). After rinsing, sections were incubated with goat anti-rabbit IgG (10 nm) or rabbit anti-goat IgG (5 nm) gold conjugates from British BioCell International (BBI, Cardiff, UK). Slices were finally washed with PBS and distilled water and left to air dry. Isotype controls with identical staining procedures, but replacing the primary antibodies with negative controls (Rabbit IgG Ab-1, NC-100-P1, Neo Markers, Fremont, CA, USA and Goat IgG-UNLB, 0109-01, Southern Biotech, Birmingham, AL, USA) showed no labeling. Additional negative controls, replacing the primary antibodies with PBS were performed on every experimental day.

Imaging of the sections was performed as described above. For each nucleus, the entire 2D cross section of nuclear envelope was visualized by merging consecutive high magnification images (see Figure S8). For quantitative analyses of SERCA2a staining, the number of gold particles within a narrow band at distances of <50 nm from the midline between the inner and the outer nuclear membrane was assessed.
## Supplemental Tables

### Supplemental Table 1:

Basic characteristics of patients who underwent transplantation or heart donors

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age, y</th>
<th>EF, %</th>
<th>Meds</th>
<th>Sex</th>
<th>Age, y</th>
<th>EF, %</th>
<th>Meds</th>
<th>Sex</th>
<th>Age, y</th>
<th>EF, %</th>
<th>Meds</th>
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<tr>
<td>f</td>
<td>57</td>
<td>65</td>
<td>CAT</td>
<td>f</td>
<td>76</td>
<td>45</td>
<td>-</td>
<td>m</td>
<td>20</td>
<td>15</td>
<td>ACEI, BB</td>
</tr>
<tr>
<td>m</td>
<td>46</td>
<td>60</td>
<td>AB, PP</td>
<td>f</td>
<td>64</td>
<td>65</td>
<td>-</td>
<td>f</td>
<td>25</td>
<td>10</td>
<td>ACEI, BB</td>
</tr>
<tr>
<td>f</td>
<td>57</td>
<td>55</td>
<td>CAT</td>
<td>m</td>
<td>54</td>
<td>55</td>
<td>BB</td>
<td></td>
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</tbody>
</table>

Data are mean±SD. EF – ejection fraction; Meds – medications; CAT – catecholamine; AB – antibiotics; PP – phenprocoumon; ACEI – ACE inhibitors; BB – β-blockers; D – diuretics; ARB – angiotensin II receptor blocker.

### Supplemental Table 2:

Left ventricular echocardiographic parameters in control and 1 and 6 weeks after TAC intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham 1 week (N=16)</th>
<th>TAC 1 week (N=14)</th>
<th>TAC 6 weeks (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>510±20</td>
<td>483±21</td>
<td>510±13</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.81±0.06</td>
<td>3.66±0.10</td>
<td>4.53±0.22</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.30±0.09</td>
<td>2.38±0.10</td>
<td>3.57±0.38</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.72±0.02</td>
<td>0.95±0.03*</td>
<td>1.18±0.06</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>0.65±0.01</td>
<td>0.86±0.04*</td>
<td>1.12±0.06</td>
</tr>
<tr>
<td>FS (%)</td>
<td>40±2</td>
<td>35±2</td>
<td>18±4*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>70±2</td>
<td>65±3</td>
<td>36±8*</td>
</tr>
<tr>
<td>Rel. WT (mm/mm)</td>
<td>0.36±0.01</td>
<td>0.50±0.02*</td>
<td>0.52±0.03*</td>
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<tr>
<td>LV mass (mg)</td>
<td>71±3</td>
<td>121±8*</td>
<td>237±18*</td>
</tr>
</tbody>
</table>

Data are mean±S.E.M., *P<0.05 vs. Sham 1 week, #P<0.05 vs. TAC 1 week.
Supplemental Table 3:

<table>
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<tr>
<th>Normal Tyrode</th>
<th>Sham 1 week (n=12)</th>
<th>TAC 1 week (n=12)</th>
<th>TAC 7 weeks (n=10)</th>
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<tr>
<td></td>
<td>cytoplasm</td>
<td>nucleus</td>
<td>cytoplasm</td>
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<tr>
<td>Diastolic [Ca(^{2+})] (nM)</td>
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<tr>
<td>0.5 Hz</td>
<td>88 ± 5</td>
<td>126 ± 4</td>
<td>112 ± 10</td>
</tr>
<tr>
<td>1 Hz</td>
<td>96 ± 6(^*)</td>
<td>151 ± 7(^*)</td>
<td>134 ± 8(^*)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>118 ± 5(^*)</td>
<td>207 ± 9(^*)</td>
<td>175 ± 9(^{*\dagger})</td>
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<tr>
<td>5 Hz</td>
<td>199 ± 11(^*)</td>
<td>379 ± 9(^*)</td>
<td>309 ± 35(^{*\dagger})</td>
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<td>Systolic [Ca(^{2+})] (nM)</td>
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<tr>
<td>0.5 Hz</td>
<td>687 ± 50</td>
<td>499 ± 45</td>
<td>711 ± 45</td>
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<td>1 Hz</td>
<td>724 ± 46(^*)</td>
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<td>2 Hz</td>
<td>795 ± 56(^*)</td>
<td>639 ± 32(^*)</td>
<td>779 ± 35</td>
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<tr>
<td>5 Hz</td>
<td>868 ± 63(^*)</td>
<td>734 ± 41(^*)</td>
<td>797 ± 29</td>
</tr>
<tr>
<td>Amplitude (nM)</td>
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<td>0.5 Hz</td>
<td>599 ± 46</td>
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<td>1 Hz</td>
<td>628 ± 41</td>
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<td>596 ± 52</td>
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<tr>
<td>2 Hz</td>
<td>677 ± 54(^*)</td>
<td>432 ± 35(^*)</td>
<td>604 ± 40</td>
</tr>
<tr>
<td>5 Hz</td>
<td>669 ± 53</td>
<td>355 ± 42</td>
<td>488 ± 58(^{*\dagger})</td>
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<tr>
<th>Isoprenaline</th>
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<tr>
<td>Diastolic [Ca(^{2+})] (nM)</td>
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<tr>
<td>0.5 Hz</td>
<td>221 ± 10</td>
<td>232 ± 13</td>
<td>253 ± 20</td>
<td>275 ± 23</td>
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<td>286 ± 21</td>
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<tr>
<td>1 Hz</td>
<td>222 ± 10</td>
<td>251 ± 18</td>
<td>270 ± 20</td>
<td>364 ± 24(^{*\dagger})</td>
<td>288 ± 18(^{*\dagger})</td>
<td>403 ± 34(^{*\dagger})</td>
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<tr>
<td>2 Hz</td>
<td>223 ± 10</td>
<td>292 ± 19(^*)</td>
<td>300 ± 22(^{*\dagger})</td>
<td>508 ± 26(^{*\dagger})</td>
<td>348 ± 27(^{*\dagger})</td>
<td>565 ± 32(^{*\dagger})</td>
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<tr>
<td>5 Hz</td>
<td>225 ± 10</td>
<td>375 ± 24(^*)</td>
<td>499 ± 19(^{*\dagger})</td>
<td>726 ± 28(^{*\dagger})</td>
<td>602 ± 44(^{*\dagger})</td>
<td>893 ± 54(^{*\dagger})</td>
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<td>Systolic [Ca(^{2+})] (nM)</td>
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<tr>
<td>0.5 Hz</td>
<td>1742 ± 26</td>
<td>1556 ± 36</td>
<td>1527 ± 75(^\dagger)</td>
<td>1187 ± 91(^\dagger)</td>
<td>1122 ± 75(^\dagger)</td>
<td>991 ± 26(^\dagger)</td>
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<td>1 Hz</td>
<td>1743 ± 29</td>
<td>1551 ± 41</td>
<td>1533 ± 82</td>
<td>1207 ± 98(^\dagger)</td>
<td>1173 ± 80(^\dagger)</td>
<td>1017 ± 42(^\dagger)</td>
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<tr>
<td>2 Hz</td>
<td>1750 ± 28</td>
<td>1568 ± 43</td>
<td>1541 ± 87</td>
<td>1225 ± 112(^\dagger)</td>
<td>1189 ± 80(^\dagger)</td>
<td>1001 ± 16(^\dagger)</td>
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<tr>
<td>5 Hz</td>
<td>1754 ± 28</td>
<td>1577 ± 53</td>
<td>1518 ± 94(^\dagger)</td>
<td>1252 ± 125(^\dagger)</td>
<td>1155 ± 74(^\dagger)</td>
<td>1016 ± 37(^\dagger)</td>
</tr>
<tr>
<td>Amplitude (nM)</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>0.5 Hz</td>
<td>1521 ± 32</td>
<td>1325 ± 36</td>
<td>1273 ± 67(^\dagger)</td>
<td>912 ± 82(^\dagger)</td>
<td>858 ± 58(^\dagger)</td>
<td>705 ± 27(^\dagger)</td>
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<tr>
<td>1 Hz</td>
<td>1522 ± 35</td>
<td>1300 ± 43</td>
<td>1263 ± 71(^\dagger)</td>
<td>843 ± 83(^\dagger)</td>
<td>885 ± 66(^\dagger)</td>
<td>614 ± 36(^\dagger)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>1527 ± 32</td>
<td>1277 ± 43</td>
<td>1242 ± 75(^\dagger)</td>
<td>717 ± 111(^{*\dagger})</td>
<td>841 ± 60(^{*\dagger})</td>
<td>435 ± 38(^{*\dagger})</td>
</tr>
<tr>
<td>5 Hz</td>
<td>1529 ± 33</td>
<td>1202 ± 49(^*)</td>
<td>1018 ± 81(^{*\dagger})</td>
<td>526 ± 127(^{*\dagger})</td>
<td>553 ± 54(^{*\dagger})</td>
<td>123 ± 29(^{*\dagger})</td>
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Data are mean±S.E.M., *P<0.05 vs. previous frequency, †P<0.05 vs. Sham 1 week.
**Supplemental Figures**

**Supplemental Figure 1.** Characterisation of pressure overload-induced hypertrophy in adult WT mice. (a) LVEDD (dilation) in conjunction with increased LV thickness (hypertrophy), measured as the sum of intraventricular septum thickness and posterior wall thickness for sham (N=16) and TAC-operated mice (after 1 week N=14 and after 6 weeks N=5). Heart weight normalized to left tibia length (N=5-6 per group) or body weight (N=6-10 per group) are shown in (b) and (c), respectively. Asterisks indicate P<0.05 versus 1 week sham controls.
**Supplemental Figure 2.** Nucleus size and number of tubular structures after pressure overload-induced hypertrophy in mouse cardiomyocytes and in cardiomyocytes from non-failing and failing rabbit and human hearts. Nuclear length (*left*), width (*middle*) and number of tubular invaginations per nucleus (*right*) from mouse (*top*), rabbit (*middle*) and human (*bottom*) hearts. Data from a total of 90 myocytes per group for mouse, 30 myocytes per group for rabbit and 20 myocytes per group for human. Asterisks indicate P<0.05 versus 1 week sham or non-failing controls.
Supplemental Figure 3. Expression and localization of RyR after pressure overload-induced hypertrophy in mouse cardiomyocytes. Original 2D confocal images of 2 example cardiomyocytes isolated from sham- (left) and TAC-operated mice (right) 7 weeks after the intervention and double staining with an anti-RyR antibody and DAPI to visualize the nuclei.
**Supplemental Figure 4.** Quantification of SERCA2a on the nuclear envelope of cardiomyocytes isolated from non-failing and failing murine hearts.

For each nucleus, the entire 2D cross section of the NE was visualized by merging consecutive high magnification images, as illustrated here, allowing counting of gold particles (highlighted as red dots or indicated with red arrows for more comprehensive representation) and measuring NE circumference. Gold particles were counted within a distance of <50 nm from the midline between the inner and the outer nuclear membrane.
Supplemental Figure 5. Expression of Ca\textsuperscript{2+}-regulating proteins in isolated human cardiac nuclei.

(a) Immunoblots of the various fractions of the protocol used for isolation of the nuclei from cardiac tissue. Nucleoporin 62 (Nup62) and Nkx2.5 served as nuclear markers, whereas GAPDH served as cytoplasmic marker. H – tissue homogenate; S1 and 2 – the first and the second supernatant; P1 and 2 – the first and the second pellet; N - the final purified nuclear fraction (N). (b) Original 2D transmitted light (top), Syto-16 fluorescence (bottom) and merged (middle) image of a nucleus from the final purified nuclear fraction. (c) Example immunoblots of heart homogenate (H) and purified nuclear fraction (N) from non-failing and failing human myocardium stained for RyR, IP\textsubscript{3}R and SERCA2a. For quantification, signals were normalized to GAPDH (H) or Ponceau staining (H and N). (d) Change in expression levels of Ca\textsuperscript{2+} release channels, RyR and IP\textsubscript{3}R, and SERCA in the nuclear fractions of left ventricles (red) and left ventricular tissue homogenates (black) from end-stage failing human myocardium as compared to non-failing (dashed line). Asterisks indicate P<0.05 versus non-failing controls.
Supplemental Figure 6. Hypertrophy-induced increase in diastolic [Ca\(^{2+}\)] in the nucleus versus cytoplasm of mouse and human ventricular myocytes. Increase in diastolic [Ca\(^{2+}\)] calculated as the difference to sham-operated mice or non-failing human hearts. Data from a total of 15 mouse and 6-10 human ventricular myocytes. Asterisks indicate P<0.05 versus 1 week post-TAC or moderately failing group and pound signs indicate P<0.05 versus cytoplasm.
Supplemental Figure 7. Characterisation of subcytoplasmic CaTs after pressure overload-induced hypertrophy in electrically stimulated mouse cardiomyocytes. (a) Line scan confocal imaging of subcytoplasmic CaTs in an electrically stimulated ventricular cardiomyocyte. (b) Averaged CaTs of distinct subcellular regions, as indicated in the schematic representation of the cell in (A): central (blue) versus subsarcolemmal region (black) from Sham-operated animals (left) and TAC-operated animals isolated 1 (middle) and 7 (right) weeks after the intervention. (c) Diastolic [Ca^{2+}] and (d) kinetic parameters (time to peak (left) and DT_{50} (right)) of the central (blue) and subsarcolemmal (black) CaTs. Data in (b) to (d) from a total of 10 mouse ventricular myocytes per group. Asterisks indicate P<0.05 versus 1 week Sham controls and pound signs indicate P<0.05 versus subsarcolemmal.
Supplemental Figure 8. Characterisation of cytoplasmic and nucleoplasmic CaTs of cardiomyocytes isolated from non-failing and failing rabbit hearts.

(a) Averaged original recordings of electrically stimulated CaTs in the nucleus (red) versus cytoplasm (black) of ventricular cardiomyocytes from non-failing (N=3, left) and failing (N=3, right) rabbit hearts. (b) Diastolic [Ca\(^{2+}\)], (c) amplitude and (d) kinetic parameters (time to peak (left) and DT\(_{50}\) (right)) of the nucleoplasmic (red) and cytoplasmic (black) CaTs. Data in (a-d) from a total of 15 myocytes per group. Asterisks indicate P<0.05 versus non-failing group.
References


