Mechanisms of reduced contractility in an animal model of hypertensive heart failure

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Summary

1. Alterations in intracellular Ca^{2+} homeostasis have frequently been implicated as underlying the contractile dysfunction of failing hearts. Contraction in cardiac muscle is due to a balance between sarcolemmal (SL) and sarcoplasmic reticulum (SR) Ca^{2+} transport which has been studied in single cells and small tissue samples. However, many studies have not used physiological temperatures and pacing rates, and this could be problematic given different temperature dependencies and kinetics for transport processes.

2. The spontaneously hypertensive rat (SHR) and their age-matched Wistar Kyoto controls (WKY) provide an animal model of hypertensive failure with many features in common to heart failure in humans. Steady-state measurements of Ca^{2+} and force showed peak stress was reduced in trabeculae from failing SHR hearts in comparison to WKY, although the Ca^{2+} transients were bigger, and decayed more slowly.

3. Dynamic Ca^{2+} cycling was investigated by determining the recirculation fraction (RF) of activator Ca^{2+} through the SR between beats during recovery from experimental protocols that potentiated twitch force. No difference in RF between rat strains was found, although the RF was dependent on the potentiation protocol used.

4. Superfusion with 10 mM caffeine and 0 mM $[Ca^{2+}]_o$ was used to measure SL Ca^{2+} extrusion. The caffeine-induced $[Ca^{2+}]_i$ transient decayed more slowly in SHR trabeculae, suggesting SL Ca^{2+} extrusion was slower in SHR.

5. Ultrastructural immunohistochemical analysis of left ventricular (LV) free wall sections using confocal microscopy showed that t-tubule organization was disrupted in myocytes from SHR, with reduced labelling of the SR Ca^{2+}-ATPase (SERCA2a) and Na^+-Ca^{2+} exchanger (NCX) in comparison to WKY, with the latter possibly related to a lower fraction of t-tubules per unit cell volume.

6. We suggest that although Ca^{2+} transport is altered in the progression to heart failure, force development is not limited by the amplitude of the Ca^{2+} transient. Despite slower SR Ca^{2+} transport, the recirculation fraction and dynamic response to a change of inotropic state minimally altered changes in the SHR model because there was a similar slowing in Ca^{2+} extrusion across the surface membrane.

Introduction

Changes in intracellular $[Ca^{2+}]$ play a pivotal role in regulation of contraction and relaxation in the heart. Depolarization during the action potential results in SL Ca^{2+} entry *via* voltage-gated Ca^{2+}-channels that subsequently triggers the release of a larger amount of Ca^{2+} from the sarcoplasmic reticulum (SR), in a process known as Ca^{2+}-induced Ca^{2+}-release.[1] Further Ca^{2+} entry may occur during the peak of the action potential *via* the SL Na^+-Ca^{2+} exchanger (NCX) in 'reverse' mode.[2] The increased cytosolic $[Ca^{2+}]$ allows crossbridge cycling, and force production. Relaxation occurs on removal of Ca^{2+} from the cytoplasm, *via* two principle routes: re-uptake into the SR by a Ca^{2+}-ATPase (SERCA2a),[3] or transport across the SL primarily *via* the 'forward' mode of NCX.[4] When the heart is in a steady-state, that is, when systolic and diastolic pressures are constant from beat-to-beat, this 'Ca^{2+} cycling' through intracellular and extracellular compartments is balanced so that the amount of Ca^{2+} released from the SR equals that taken up by SERCA2a, and Ca^{2+} extruded across the SL is the same as the amount that entered during systole. However, heart rate and contractility vary depending on activity, and require dynamic changes in the relative contributions of SR and SL Ca^{2+} fluxes to change the amplitude of the Ca^{2+} transient. An imbalance between influx and efflux allows a new steady state to be achieved which is re-established by the changed Ca^{2+} transient.[5]

In failing hearts, the ventricles no longer contract with sufficient force to produce the required cardiac output. Alterations in Ca^{2+} cycling are often implicated in the patho-physiology of heart failure.[6-9] Many studies on isolated myocytes from animal models of heart failure report that the amplitude of the Ca^{2+} transients is decreased,[10-12] and infer that a reduced contractility will ensue. However, this inference should be treated with caution as isolated myocytes are frequently damaged by isolation, and are depotentiated by working at slack length. In addition, the removal of the extracellular matrix may lead to defects in normal signalling between force and Ca^{2+} as mediated by stretch activated Ca^{2+} influx pathways and adhesion complexes.[13,14] Nevertheless, morphological analysis of ventricular tissue from failing hearts and isolated myocytes have both shown changes to the T system that could disrupt the synchronisation of SR Ca^{2+} release and impair contractility.[15,16]

While observations of reduced Ca^{2+} transients provide a facile explanation for impaired contractility, it should be noted that for some heart failure models the

ventricles are dilated, with thin walls, suggesting (from the Law of La Place) that contractile performance should be *increased* to achieve the same systolic blood pressure (which is even higher in the case of systemic hypertension). A second potential concern is that much of our knowledge of the cellular mechanisms associated with the development of heart failure has come from animal models that have a relatively abrupt disease onset compared to human failure (*e.g.* aortic banding compared to systemic hypertension-induced heart failure). Furthermore, in human heart failure, a long period of failure is supported by drug therapy that may lead to more extensive maladaptation (*i.e.* additional changes may occur that could be poorly replicated in some animal models).

These considerations suggest that we should re-examine the relationship between force and $[Ca^{2+}]_i$ utilizing an animal model of hypertensive failure under experimental conditions that are close to physiological. We briefly describe our investigation of Ca^{2+} handling in left ventricular (LV) trabeculae from spontaneously hypertensive rats (SHR) in failure and their age-matched Wistar-Kyoto (WKY) controls.[17,18] As has been previously described,[19-21] the SHR model has many features in common with human hypertensive heart failure, including progressive cardiac hypertrophy that develops over ~18 months, leading to premature death.

Relationship between Ca^{2+} and force

Steady-state measurements of force and Ca^{2+}

Defective Ca^{2+} cycling has been reported in myocytes isolated from SHR during the period of stable hypertrophy that precedes the transition to failure at around 20 months of age.[22,23] However, only a few studies have examined Ca^{2+} and force in *failing* SHR hearts. At low stimulation frequency and at room temperature, Brooks *et al.* (1994)[24] reported a small increase in peak Ca^{2+} (measured in perfused hearts using aequorin) that did not achieve statistical significance. Although uncalibrated, they also noted a decrease in the amplitude of the aequorin transient with increasing rate, which was mirrored by force. A more recent study in whole hearts using fluo-4/AM reported changes in Ca^{2+} cycling in SHR that preceded the onset of overt heart failure.[25] This study showed an initial increase in the amplitude of the fluo-4 transient between 6 and 9 months, followed by a decrease at 22 months. However, these intriguing measurements cannot be simply translated to Ca^{2+} levels due to the non-quantitative nature of the measurement and/or lack of knowledge of changing resting Ca^{2+} levels and background fluorescence. Unfortunately, in both cases the hearts were at room temperature, which raises the question as to how the temperature sensitivity of Ca^{2+} cycling in the different experimental groups might influence their results.

To address these concerns, we have examined steady-state force and $[Ca^{2+}]_i$ in left ventricular trabeculae loaded with the ratiometric indicator fura-2/AM at physiological heart rates (for rat, 5 Hz) and at 37°C.[17,18] In these conditions, SHR trabeculae developed less stress that those from age-matched, normotensive WKY hearts (SHR: 8.5±1.4 mN mm^{-1}, WKY: 24.8±5.5 mN mm^{-1}, n=6 both groups). Both the peak of the Ca^{2+} transients (SHR: 0.77±0.09 μM, WKY: 0.44±0.08 μM), and the resting $[Ca^{2+}]_i$ were higher for SHR (SHR: 0.20±0.03 μM, WKY: 0.068±0.014 μM), and the time constant of decay in $[Ca^{2+}]_i$ slower (SHR: 0.064±0.002 s, WKY: 0.053±0.004 s). Exemplar data are shown in Figure 1 for Ca^{2+} transients and force from SHR (dashed lines) and WKY (solid lines), averaged over 16 cycles. Figure 1B shows the same data as in 1A, normalized to show the difference in the time course of the Ca^{2+} transients between rat strains.

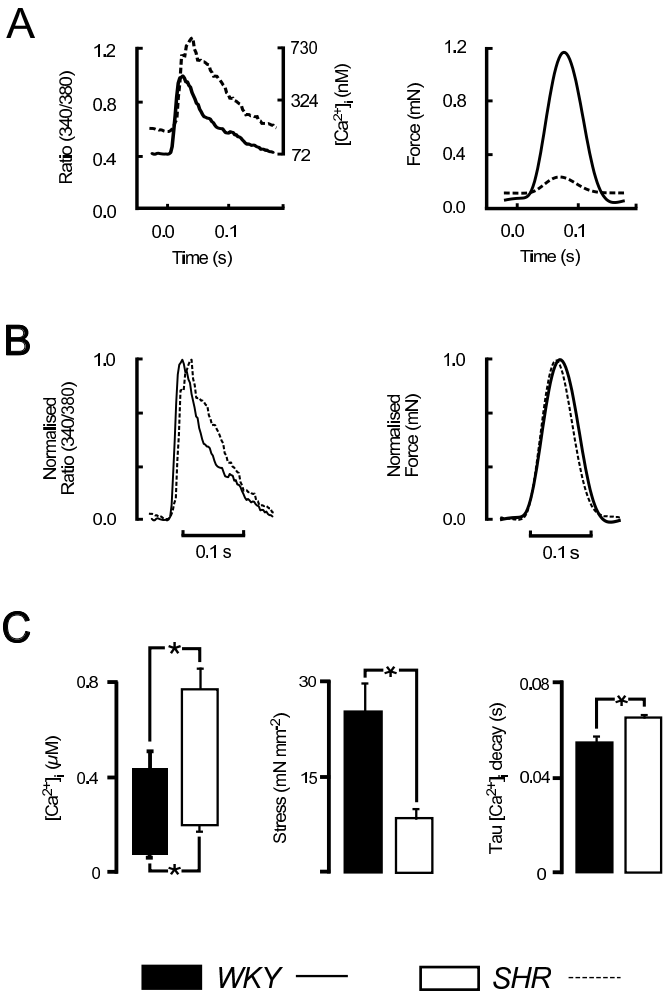

***Figure 1. $[Ca^{2+}]_i$ and force at physiological frequency**. **A:** Representative examples of $[Ca^{2+}]_i$ and force (averaged from 16 cycles) obtained from WKY (solid lines) and SHR (dashed lines) LV trabeculae of similar diameter, superimposed for comparison (5 Hz, 2 mM $[Ca^{2+}]_o$, 37°C). **B:** shows the data from A normalized to the peaks to emphasise the differences between rat strains. **C:** shows mean±S.E.M. data from WKY (solid bars) and SHR (open bars), n=6 both groups. * denotes* p≤*0.05.*

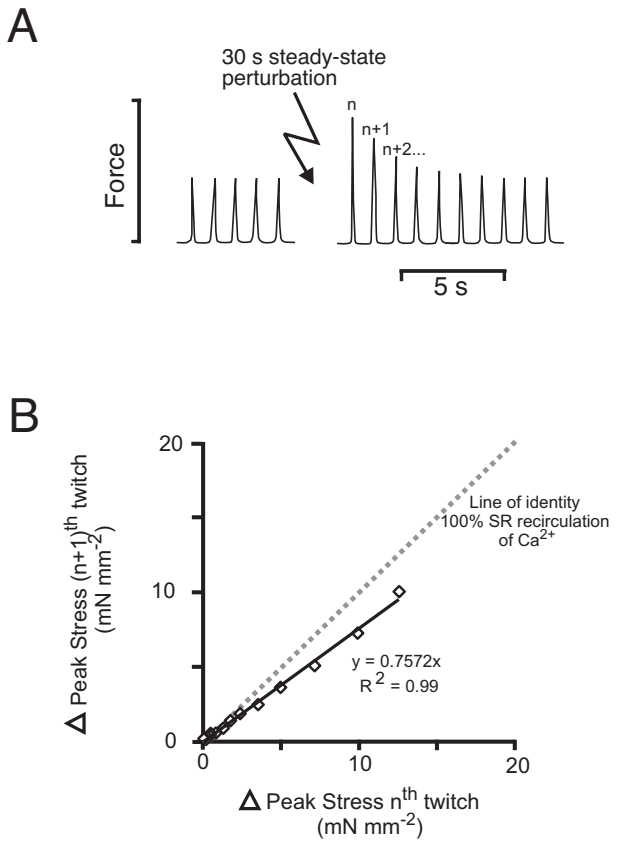

Figure 2. Beat-to-beat recirculation of activator Ca^{2+} through the SR during recovery to steady-state. *Trabeculae were stimulated at 1 Hz and subjected to a 30 s train of paired-pulses (80 ms between stimulus pairs), and after steady-state had again been reached, a 30 s rest period was used as a second potentiation protocol.* ***A:*** *shows the recovery of stress to steady-state following non-pharmacological potentiation for a representative trabecula. Estimation of the recirculation fraction for the recovery of stress following potentiation is shown in* ***B****. The peak of the $(n + 1)^{th}$ twitch (normalized to steady-state) was plotted against the peak of the n^{th} twitch, and the slope of the linear regression line fitted to the data used as an estimate of the relative amount of activator recirculated through the SR. The dotted line has unity slope, and represents the hypothetical case if all of the activator Ca^{2+} was recirculated through the SR between beats.*

Non-steady-state Ca^{+} cycling.

As described above, in steady-state, the amount of Ca^{2+} that leaves each myocyte during diastole must be quantitatively identical to the amount that entered during systole. However, to change the Ca^{2+} cycling and contractility, Ca^{2+} influx (or efflux) must change to increase (or decrease) the SR Ca^{2+} content[26] that is the major determinant of contractility. The change in SR content takes several beats to develop, and limit the speed of adaption of the working ventricle to changes in load. Experimentally, the non steady-state behaviour can be examined either by recovery from an intervention that alters intracellular Ca^{2+} levels, or by the approach to steady-state from a change in rate. In Figure 2, we illustrate results from experiments to examine non-steady-state Ca^{2+} cycling in LV trabeculae using non-pharmacological interventions to potentiate force: (i) an interval of 30s rest, and (ii) a 30s train of paired-pulses, and calculate the recirculation fraction (RF) for recovery to steady-state. In rat myocytes, the SR accumulates Ca^{2+} during a period without stimulation, leading to potentiation of both the Ca^{2+} transient and twitch force on recommencing stimulation,[27] whereas paired pulsing increases Ca^{2+} by increasing influx of Na^{+} and Ca^{2+} per unit time (equivalent to increasing the duration of the AP).

To analyze the resulting response, the amplitude of the twitches were measured during the return to steady-state (see Figure 2A). Figure 2B shows the change in peak stress from a representative trabecula, plotted as the amplitude of the $(n + 1)^{th}$ twitch against the amplitude of the preceding n^{th} twitch. The gradient of lines fitted to the data is then taken as the 'recirculation fraction' (RF) which is a measure of the fraction of cytosolic Ca^{2+} re-sequestered by the SR between beats.[28,29] Using this method of non-steady-state analysis and two different experimental protocols used to potentiate force, no difference was found in the RF between aged failing SHR and WKY controls.[18] This was despite the fact that the Ca^{2+} transient decayed more slowly in SHR as might be expected from previous reports of reduced SERCA2a activity and impaired SR Ca^{2+} uptake.[17] To explain this paradox, the relative contribution of trans-SL Ca^{2+} extrusion to the decay of the Ca^{2+} transient would have to be also decreased in SHR. This underscores the problem that, while easy to measure, the RF is rather difficult to interpret without additional evidence for the relative contribution of SL and SR calcium transport processes.

Two previous studies have also examined non-steady-state Ca^{2+} handling in SHR. Both Pérez *et al.* (1993)[30] and Lammerich *et al.* (1995)[31] found SR Ca^{2+} release was reduced in papillary muscles from young SHR, and that the RF was decreased. This is clearly at variance with the results described above, but we note that the latter studies were performed on young animals (~6 months old, compared to ~22 months in our study[18]) and it is possible that changes in the first stages of heart failure may not reflect the situation after a long period of adaption to hypertension (as would be the case for human hypertension, and see also Kapur *et al.*, 2010[25]). This consideration suggests that changes in SR Ca^{2+} transport may occur at an earlier stage of disease in SHR than mechanisms that slow SL transport.

Measuring trans-sarcolemmal Ca^{2+} fluxes

Sarcolemmal Ca^{2+} fluxes can be measured by addition of 10 mM caffeine to the superfusate. In quiescent myocytes, this causes the release of Ca^{2+} from the SR and a transient increase in $[Ca^{2+}]_i$. In the continued presence of caffeine, the $[Ca^{2+}]_i$ decays slowly to resting levels as the SL Ca^{2+} transport proteins extrude Ca^{2+}. By also removing

external Ca^{2+}, any entry of Ca^{2+} is abolished so that unidirectional efflux can be determined and hence SL Ca^{2+} transport. Figure 3A shows fluorescence from a representative trabecula at 37°C before, during, and after caffeine superfusion. 3B shows mean data for the time constant of decay of the caffeine transients, suggesting that SL Ca^{2+} extrusion was slower in SHR (SHR: 13.3±1.8 s, WKY: 8.6±1.5 s, n=3 both groups). With this result in mind, we can now reconcile the slowed Ca^{2+} transients in SHR in the absence of any change in RF.

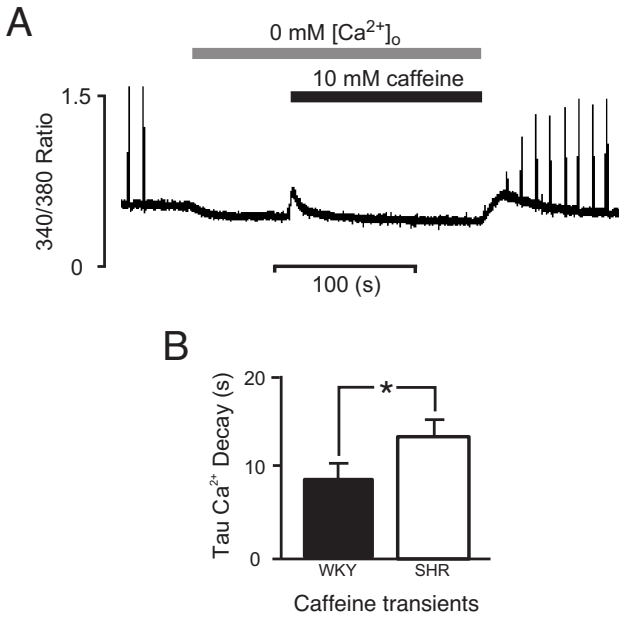

Figure 3. Sarcolemmal Ca^{2+} transport. *Measurement of SL Ca^{2+} transport was made in quiescent trabeculae by prolonged application of 10 mM caffeine in the absence of external $[Ca^{2+}]_o$. These experimental conditions deplete the SR of Ca^{2+}, causing a 'transient' increase in $[Ca^{2+}]_i$ that then slowly decays as Ca^{2+} is extruded from the myocytes, primarily by the SL NCX.* ***A:*** *shows an example of fluorescence data from a trabecula superfused with 10 mM caffeine in 0 mM $[Ca^{2+}]_o$. An exponential fitted to the decay phase of the caffeine transient (from 90%-10% of the peak $[Ca^{2+}]_i$) was used as a measure of SL Ca^{2+} transport.* ***B:*** *shows mean±S.E.M. time constant of decay from WKY (filled, n=3) and SHR (open, n=3). * denotes* p≤*0.05.*

Localization of Ca^{2+} transporters

Immunohistochemistry of SERCA2a, NCX and WGA

Song *et al.* (2006),[15] using isolated myocytes from failing SHR hearts, showed decreased Ca^{2+} transients which they attributed to t-tubular remodelling (for review see Louch, Sejersted & Swift, 2010[32]). T-tubular remodelling has also been observed in human,[16] pig,[33] and other models of heart failure.[33] Since we did not observe a decrease in the Ca^{2+} transient it was therefore of interest to see the extent to which sub-cellular changes mimic those of Song *et al.*

Immunohistological examination of ventricular tissue from SHR and WKY was carried out to compare the localization of NCX and SERCA2a and the subcellular distribution of t-tubules between SHR and control hearts. Figure 4A shows the analysis of t-tubule structure (top panels) using a skeletonization algorithm (lower panels). Figure 4B shows images of typical antibody-labelling patterns for SERCA2a (red) and NCX (green) in WKY and SHR ventricular cells. Quantification (Figure 4C) showed SERCA2a (SHR: 79.6±7.6 % WKY, $p \leq 0.05$) and NCX (SHR: 63.6±6.2% WKY, $p \leq 0.05$) were both decreased when expressed as a percentage of WKY label (3 cells/heart, n=3 hearts per group). Figure 4C (i) showed t-tubule area was reduced in SHR, with some areas showing marked disruption (WKY: 4.3±0.2 % area, SHR: 3.2±0.3 % area, $p \leq 0.05$).

Conclusion

As reported previously (*e.g.* Ward *et al.*, 2003[17]) trabeculae from failing SHR hearts developed reduced force (stress) in comparison to age-matched WKY controls at stimulation frequencies that encompassed the physiological range. Although there are numerous reports of changes in the amplitude of the Ca^{2+} transient in isolated myocytes which have been implicated in the reduced force response of failing hearts[11] we found this did not appear to be the case in the intact ventricular trabeculae. On the other hand, there were differences in the time course of the Ca^{2+} transient between rat strains with the SHR having a slower decline in Ca^{2+}, in agreement with other studies,[34,35] and we found similar subcellular remodeling.[18] Since the rate of decline of the Ca^{2+} transient is dominated by the activity of SERCA2a,[3,26] one might expect a reduced SR Ca^{2+} content in SHR (and recirculation fraction), an explanation that has been invoked previously to explain a reduced Ca^{2+} transient amplitude in HF. In support of the idea that reduced SR uptake is present in the SHR, we also note that diastolic Ca^{2+} is elevated in SHR (see Figure 1A).[17]

An explanation for this apparent paradox is provided by our caffeine experiments which showed a parallel decrease in SL Ca^{2+} extrusion. Since NCX is the major SL extrusion mechanism, our observation of reduced NCX labeling seems a simple explanation for why recirculation fraction is unchanged and why the SR store is not depleted despite reduced SERCA2a expression (Figure 4).[36] This change was accompanied by a reduction in SL surface membrane area in t-tubules. In addition, the reduced stress was not associated with reduced Ca^{2+} transients, although differences in $[Ca^{2+}]_i$ were evident between rat strains: the Ca^{2+} transients decayed more slowly in SHR and resting $[Ca^{2+}]_i$ was increased. The delayed return to resting levels apparent in SHR provides an explanation for the diastolic dysfunction observed in heart failure.[34,37,38] Although we have provided an explanation for why the Ca^{2+} transient amplitude can be unaltered in the presence of reduced SR uptake rate, the cause of the loss of force remains unclear. We have previously shown that there is an increase in collagen which may impede external force production,[17] but changes in cross bridge cycling and changes in troponin

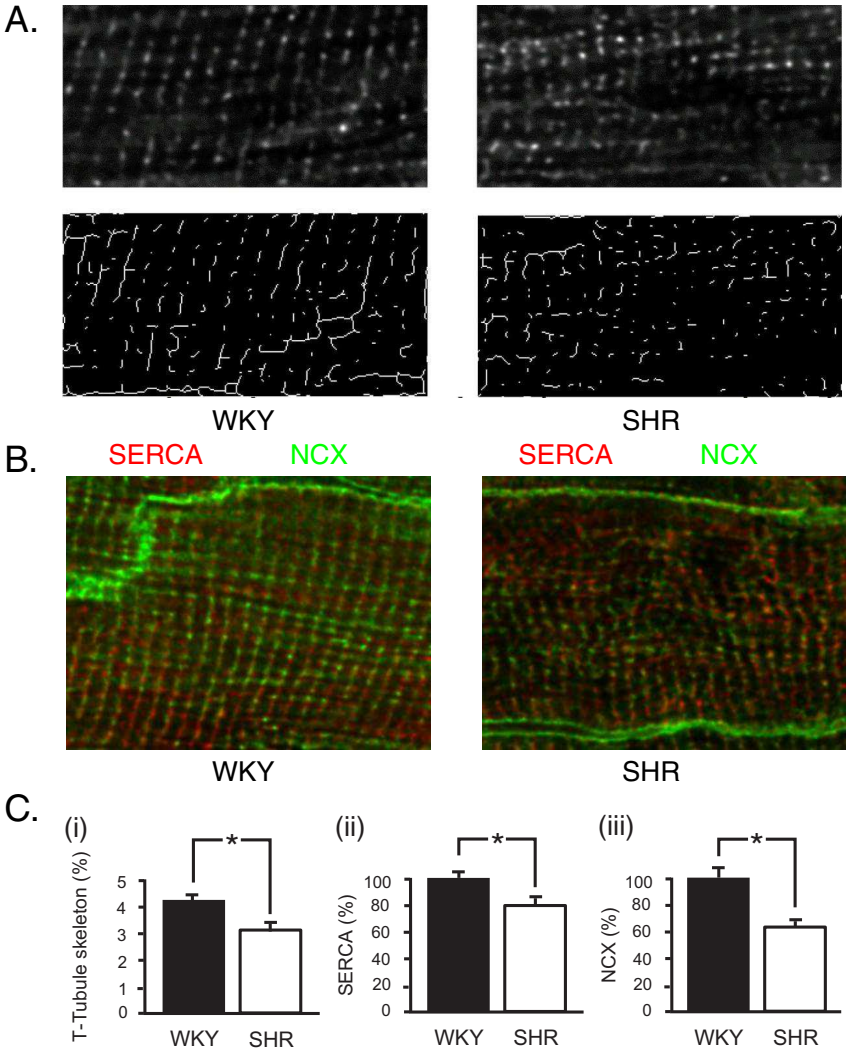

Figure 4. Confocal imaging of t-tubules, SERCA2a and NCX in LV free wall. *Tissue sections of LV free wall were fluorescently labeled for t-tubules (WGA), SERCA2a and NCX and imaged with confocal microscope at 0.1 μm × 0.1 μm × 1 μm pixel resolution.* ***A:*** *The top panels show representative myocytes from WKY and SHR labeled with WGA. The lower panel shows the result of processing with an automated skeletonization algorithm (Image J).* ***B:*** *representative images taken of LV myocytes from free wall sections labelled with antibodies raised against SERCA2a (red) and NCX (green).* ***C:*** *histograms of (i) pixels after skeletonization of the WGA image (left panel), (ii) SERCA2a (middle panel) and (iii) NCX (right panel), expressed as a percentage of the WKY labelling. Data from 3 cells/heart, n = 3 hearts per group. Analysis showed t-tubule area was reduced in SHR, with some areas showing marked disruption (WKY: 4.3 ± 0.2% area, SHR: 3.2±0.3% area). * indicates* $p \leq 0.05$.

sensitivity may also occur.[39-41] Nevertheless, it is important to note that in the intact working heart, such a reduction in force is unlikely to be present (a conclusion due to the maintenance of systolic pressure in the presence of ventricular dilation) and must therefore be offset by a further increase in the amplitude of the Ca^{2+} transient, possibly due to increased β-adrenergic drive.

Acknowledgements

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