Angiotensin II–Mediated Adaptive and Maladaptive Remodeling of Cardiomyocyte Excitation–Contraction Coupling

Konstantin Gusev, Andrea A. Domenighetti, Lea M.D. Delbridge, Thierry Pedrazzini, Ernst Niggli, Marcel Egger

Abstract—Cardiac hypertrophy is associated with alterations in cardiomyocyte excitation–contraction coupling (ECC) and Ca$^{2+}$ handling. Chronic elevation of plasma angiotensin II (Ang II) is a major determinant in the pathogenesis of cardiac hypertrophy and congestive heart failure. However, the molecular mechanisms by which the direct actions of Ang II on cardiomyocytes contribute to ECC remodeling are not precisely known. This question was addressed using cardiomyocytes isolated from transgenic (TG1306/1R [TG]) mice exhibiting cardiac specific overexpression of angiotensinogen, which develop Ang II–mediated cardiac hypertrophy in the absence of hemodynamic overload. Electrophysiological techniques, photolysis of caged Ca$^{2+}$ and confocal Ca$^{2+}$ imaging were used to examine ECC remodeling at early (~20 weeks of age) and late (~60 weeks of age) time points during the development of cardiac dysfunction. In young TG mice, increased cardiac Ang II levels induced a hypertrophic response in cardiomyocyte, which was accompanied by an adaptive change of Ca$^{2+}$ signaling, specifically an upregulation of the Na$^{+}$/Ca$^{2+}$ exchanger–mediated Ca$^{2+}$ transport. In contrast, maladaptation was evident in older TG mice, as suggested by reduced sarcoplasmic reticulum Ca$^{2+}$ content resulting from a shift in the ratio of plasmalemmal Ca$^{2+}$ removal and sarcoplasmic reticulum Ca$^{2+}$ uptake. This was associated with a conserved ECC gain, consistent with a state of hypersensitivity in Ca$^{2+}$–induced Ca$^{2+}$ release. Together, our data suggest that chronic elevation of cardiac Ang II levels significantly alters cardiomyocyte ECC in the long term, and thereby contractility, independently of hemodynamic overload and arterial hypertension. (Circ Res. 2009;105:42-50.)

Key Words: cardiac excitation–contraction coupling ■ remodeling ■ sodium–calcium exchange ■ angiotensin II ■ hypertrophy

Hypertension is a major risk factor for the genesis and progression of a variety of cardiovascular diseases including heart failure, stroke, and kidney dysfunction. Common therapies for the treatment of hypertension lower blood pressure via inhibition of the renin–angiotensin system (RAS). Drugs include inhibitors of the angiotensin-converting enzyme, as well as angiotensin II (Ang II) receptor type 1 (AT$_1$) antagonists, thus preventing the formation of Ang II or its binding to AT$_1$ receptors.$^{1,2}$ In addition to its effects on the vasculature, the RAS plays an important role in the development of cardiac hypertrophy.$^{3}$ Sustained activation of the RAS results in arterial hypertension, which in turn produces hemodynamic overload. The adaptation of the heart to this loading is characterized by a change in cardiac protein expression and substantial cardiac remodeling. In addition, Ang II also exerts direct growth-promoting effects on cardiac tissues, resulting in cardiomyocyte hypertrophy and mechanical dysfunction independently of hypertension.$^{4,5}$ Ang II activates several intracellular signal transduction pathways including mitogen-activated protein kinases and protein kinase C.$^{6-7}$ Therefore, a local increase in the Ang II concentration can contribute significantly to the pathogenesis of cardiac hypertrophy, even in the absence of arterial hypertension.$^{8}$

Cardiomyocyte excitation–contraction coupling (ECC) involves Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) initiated by membrane depolarization and subsequent Ca$^{2+}$ influx via voltage-activated L-type Ca$^{2+}$ channels. This triggers further Ca$^{2+}$ release via SR-Ca$^{2+}$ release channels (ryanodine receptors [RyRs]), an amplification mechanism known as Ca$^{2+}$–induced Ca$^{2+}$ release (CICR). The activation of a cluster of RyRs by individual L-type Ca$^{2+}$ channel openings produces localized Ca$^{2+}$ release events (Ca$^{2+}$ sparks), the building block of the global Ca$^{2+}$ transients. Cardiac hypertrophy is often associated with alterations in ECC and Ca$^{2+}$ handling.$^{9-11}$ However, the precise molecular
mechanisms and the temporal aspects of shifts in Ca\(^{2+}\) homeostasis are not well understood and may depend on the initial cause of the hypertrophic response.\(^{12}\) Supporting this notion, the expression levels and the function of several Ca\(^{2+}\) signaling proteins have been found to be differently altered in various forms of hypertrophy.\(^{13-16}\) Despite their potential importance, the effects of chronically elevated levels of intracardiac Ang II on ECC remodeling and Ca\(^{2+}\) signaling during development of hypertrophy and congestive heart failure have not been investigated.

In the present study, we examined the role of Ang II in the remodeling of cardiac ECC using the TG1306/1R transgenic (TG) mouse model. TG mice carry multiple copies of the rat angiotensinogen gene under control of the cardiac-specific \(\alpha\)-myosin heavy chain promoter, leading to elevated cardiac angiotensinogen levels. As a consequence, Ang II concentrations in the heart of TG mice are increased whereas plasma Ang II level, and therefore blood pressure, remain unchanged. Thus, the mice develop Ang II–mediated cardiac hypertrophy in the absence of high blood pressure.\(^{8}\) We have previously shown that young TG mice \(\approx 20\) weeks of age have preserved systolic function whereas old TG mice \(\approx 60\) weeks of age develop cardiomyopathy characterized by diastolic and systolic dysfunction, with a major proportion of animals also developing cardiac dilation and arrhythmias.\(^{5,18}\) We have used the TG model to interrogate the temporal impacts of chronic endogenous Ang II overproduction on cardiac Ca\(^{2+}\) signaling. We show that at early stages of disease development, increased cardiac Ang II levels induce a hypertrophic response in cardiomyocytes, accompanied by an adaptive change in Ca\(^{2+}\) signaling involving upregulation of the Na\(^+\)/Ca\(^{2+}\) exchange (NCX)-mediated Ca\(^{2+}\) transport. In contrast, at a later stage, maladaptation is evident, where the adaptive changes resulted in a damaging outcome. This is characterized by a diminished rate of NCX-mediated Ca\(^{2+}\) transport and reduced SR-Ca\(^{2+}\) content attributable to shifts in the ratio in NCX and SERCA2a function. Because ECC gain is conserved in this maladaptive situation, it suggests that a state of hypersensitivity in cardiomyocyte CICR develops at a late stage of the disease and may represent a primary ECC lesion in the progression to heart failure.

**Materials and Methods**

The TG1306/1R mouse line used for this study has been previously described.\(^{4,8,17}\) Mice (male) were studied at \(\approx 20\) weeks (young TG mice) and \(60\) weeks of age (old TG mice). All animal-handling procedures were performed with permission of the State Veterinary Administration and in accordance with the guidelines of the Swiss Animal Protection law. Cardiac ventricular myocytes were isolated from wild-type (WT) and TG mice using established enzymatic procedures.\(^{18}\) ECC machinery was studied in acute myocyte preparations or permeabilized cells using confocal microscopy in combination with or without UV flash photolysis of caged Ca\(^{2+}\) and \(I_{\text{NCX}}\) measurements in the whole-cell configuration of the patch-clamp technique as previously described.\(^{19}\) In addition, protein expression was measured by Western blotting and mRNA expression levels were assessed by real-time PCR. An expanded Materials and Method section is available in the Online Data Supplement at http://circres.ahajournals.org.

Data were reported as means \(\pm\) SEM. Student’s unpaired \(t\) test was applied to test for significance (\(P<0.01\) or \(P<0.05\), as indicated in the figure legends). For mRNA data, 1-way ANOVA \(t\) test was used to test for significance.

**Results**

**Phenotype in Young TG Mice: Calcium Currents and Excitation–Contraction Coupling Gain**

As previously reported, young TG mice demonstrated significant cardiac and cardiomyocyte hypertrophy \(\approx 20\) weeks of age.\(^{4}\) Indeed, cell surface and morphological parameters indirectly measured by membrane cell capacitance (\(C_{m}\)), were significantly increased (\(\approx 30\%\)) in TG myocytes, indicating that myocytes isolated from the left ventricular wall showed hypertrophy (Online Table I). In addition, the average calculated myocyte volume was increased significantly in TG animals (see Online Tables I and II). The efficacy of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) in activating SR-Ca\(^{2+}\) release can be expressed as ECC gain, ie, \([\text{Ca}^{2+}]_{i}\), transient amplitude (expressed as \(\Delta F/F_0\)) normalized to the \(I_{\text{Ca,L}}\) (Figure 1b). In 20-week-old TG mice, \(I_{\text{Ca,L}}\) was increased when compared to WT cells (Figure 1a and 1b). Because several studies reported reduced ECC gain in hypertrophied cardiomyocytes, which could affect contractile function, ECC gain was examined in the range from \(-20\) to \(+60\) mV.\(^{11}\) A loading protocol of 6 prepulses from \(-40\) mV to \(+10\) mV for \(200\) ms was applied before each test pulse to ensure comparable SR-Ca\(^{2+}\) loading.
conditions. In young TG mice, the ECC gain was reduced slightly, and the magnitude of this effect likely to be of little biological significance. (Figure 1b). Using a Ca\(^{2+}\) loading protocol, the SR-Ca\(^{2+}\) content was estimated by rapid application of 20 mmol/L caffeine and integration of the elicited NCX current (\(I_{\text{NCX}}\)) (Figure 2a). Peak \(I_{\text{NCX}}\) was reduced in TG myocytes by \(\approx 27\%\), whereas SR-Ca\(^{2+}\) content was reduced by \(\approx 19\%\) (Figure 2c). In addition, caffeine-induced Ca\(^{2+}\) transients were diminished to a similar extent (Figure 2b and 2c). Western blotting demonstrated a \(\approx 50\%\) decrease in SERCA2a protein expression in the heart of young TG mice when compared to age-matched WT littermates (Figure 2d). Properties of spontaneous Ca\(^{2+}\) sparks, such as frequency and amplitude, were not significantly altered in saponin-permeabilized TG cells (Figure 2e and 2f). The Ca\(^{2+}\) spark amplitude is, at least in part, determined by the surface-to-volume ratio in TG myocytes than in WT cells (Figure 2g).

**Phenotype in Young TG Mice: Transsarcolemmal Ca\(^{2+}\) Transport**

Under steady-state conditions, the Ca\(^{2+}\) influx via \(I_{\text{Ca,L}}\) is balanced on a beat-to-beat basis by Ca\(^{2+}\) removal via NCX, whereas SR-Ca\(^{2+}\) uptake equals SR-Ca\(^{2+}\) release. Figure 3a shows the experimental protocol to examine NCX activity together with \(I_{\text{NCX}}\) and the corresponding [Ca\(^{2+}\)]_i transients. \(I_{\text{NCX}}\) was induced by UV flash photolysis of caged Ca\(^{2+}\) (DM-nitrophen) under conditions of blocked CICR (ie, in the presence of thapsigargin and ryanodine; Figure 3b). Inhibiting the NCX under these conditions eliminated \(I_{\text{NCX}}\) and greatly slowed down Ca\(^{2+}\) removal (Figure 3c). To avoid modulation of NCX inactivation by [Na\(^{+}\)], and/or catalytic activation by [Ca\(^{2+}\)], amplitude-matched pairs of Ca\(^{2+}\) transients and the resulting NCX currents obtained in a WT and TG myocyte were compared (Figure 4a and 4b).\(^{20}\) At identical Ca\(^{2+}\) signal amplitudes, myocytes from 20-week-old TG mice showed larger \(I_{\text{NCX}}\) (Figure 4b). Normalized \(I_{\text{NCX}}\) density per activating [Ca\(^{2+}\)] at \(-40\) mV was increased by \(\approx 75\%\) in TG myocytes. The corresponding Ca\(^{2+}\) transport rate (ie, \(\dot{\partial}[\text{Ca}^{2+}] / \dot{\partial}t\)) was not significantly affected (Figure 4c).

Gene expression analysis investigated by real-time PCR showed an \(\approx 5\)-fold upregulation of the NCX1.1 in the hearts of 20-week-old TG mice compared with age-matched WT (Figure 4d), suggesting that the larger NCX current was associated with an increase in transporter gene expression. Finally, Figure 5a shows a plot of normalized \(I_{\text{NCX}}\) versus Ca\(^{2+}\) transport rate and confirms that TG myocytes generate more current per reduction of [Ca\(^{2+}\)]_i. Ion transport capability of the NCX is estimated by the \(I_{\text{NCX}}/\text{voltage}\) relationship, which was found to be indistinguishable between TG and WT myocytes (Figure 5b). Importantly, comparison of cell volume and cell membrane capacitance indicated a smaller surface-to-volume ratio in TG myocytes than in WT cells (Online Table II).

**Figure 2.** SR-Ca\(^{2+}\) leak in WT and TG myocytes from young animals. a, SR-Ca\(^{2+}\) content was estimated by administration of 20 mmol/L caffeine in subsequent to a loading protocol (inset, left). b, Representative data showing averaged F/Fo of fluo-3 fluorescence and corresponding line scan images of fluo-3 fluorescence (right) and \(I_{\text{NCX}}\) together with the integration curve of \(I_{\text{NCX}}\) (bottom left). c, Averaged normalized data. d, Western blot analysis comparing SERCA2a protein levels in WT and TG hearts (left). Bar graph shows the averaged quantification of SERCA2a protein expression levels for WT and TG mice. Data are expressed in arbitrary units. e, Line scan images of spontaneous Ca\(^{2+}\) sparks recorded in skinned cardiomyocytes. f, Averaged Ca\(^{2+}\) spark properties normalized to WT (Ca\(^{2+}\) spark frequency, Ca\(^{2+}\) spark amplitude). SR-Ca\(^{2+}\) content was estimated by application of 10 mmol/L caffeine. *P<0.05 vs WT (n=6 to 10, means±SEM).
Phenotype in Old TG Mice: Differences and Similarities in ECC Compared to WT

We have previously reported that 60-week-old TG animals exhibit systolic and diastolic dysfunction. A large proportion (≈40%) of these mice demonstrate dilated cardiomyopathy. Furthermore, contractile ECC latency in isolated TG cardiomyocytes is increased. Our present data revealed that membrane capacitance ($C_m$) in old TG mice was significantly increased when compared to WT mice (Online Table II). The average calculated myocyte volume was increased by 24% in TG myocytes. Interestingly, in contrast to what was seen in 20-week-old TG mice, the surface-to-volume ratio in myocytes isolated from 60-week-old TG mice was similar to WT despite substantial cellular hypertrophy.

Moreover, central elements of the ECC machinery, specifically $I_{Ca,L}$ and ECC gain, were unaltered in older TG mice (Figure 6a and 6b). Under whole-cell conditions, using a loading protocol (Figure 7a), caffeine-induced Ca$^{2+}$ transients were reduced by 30% (Figure 7a and 7b) in TG myocytes. The decay of the caffeine-induced Ca$^{2+}$ transients ($1/\tau$) was 52.9% slower (WT: 789±177 ms; TG: 1508±412 ms; n=6; means±SEM). In addition, the corresponding $I_{NCX}$ and the calculated SR-Ca$^{2+}$ content in TG cells were reduced significantly (by 29.4%) when compared to WT (Figure 7a)

![Figure 3. Experimental approach to assess NCX function. Cell image and representative $I_{NCX}$ obtained at 40 mV induced by applying UV flashes to photolysis of caged Ca$^{2+}$ (DM-nitrophen). $I_{Ca,L}$ was subsequently activated with voltage steps (200 ms) from −40 mV to 0 mV. a through c, Traces shown from top to bottom: applied voltage protocol, averaged fluo-3 fluorescence ratio profile, line scan image, and corresponding $I_{NCX}$ and $I_{Ca,L}$ Under conditions of functioning CICR. The inset (*) shows the expanded $I_{NCX}$ given above. b, $I_{NCX}$ obtained in the presence of 10 μmol/L ryanodine and 0.5 μmol/L thapsigargin to block CICR. Under these conditions, $I_{NCX}$ only depends on the amount of photolytically released Ca$^{2+}$, and within the [Ca$^{2+}$]i increases reached under our conditions, $I_{NCX}$ is known to depend linearly on [Ca$^{2+}$].

![Figure 4. Transsarcolemmal Ca$^{2+}$ pathways. a and b, Representative $I_{NCX}$ obtained in young WT littermate control ventricular myocytes (a) and in ventricular myocytes isolated from TG mice (b). Traces shown from top to bottom: averaged [Ca$^{2+}$]i profile expressed as F/Fo of fluo-3 fluorescence, line scan images of fluo-3 fluorescence, and the corresponding $I_{NCX}$ c, Summarized data from TG (gray bars) normalized for WT littermate control myocytes (black bars). $I_{NCX}$ density was normalized to the photolytically released [Ca$^{2+}$], transient (see Online Table II), Ca$^{2+}$ transport rate by NCX (free [Ca$^{2+}$] decay, dCa$^{2+}$/dt) was calculated under conditions of matched pairs of peak [Ca$^{2+}$]i, and blocked SR-function. *P<0.05, **P<0.001 vs WT cells (n=10 to 15, means±SEM). d, Real-time PCR of the NCX1 shows 453±72% increase in NCX1 mRNA. **P<0.001 vs WT cells (n=6, means±SEM).}
SERCA2a protein levels were also measured in old TG hearts (Figure 7c). Contrasting with observations in younger animals, there was no difference in total SERCA2a protein levels between WT and TG mice at 60 weeks of age. Thus, in WT, an age-dependent downregulation of SERCA2a occurred which was not seen in the TG hearts. In the TG, SERCA2a levels were already low at 20 weeks and did not further reduce at 60 weeks. In skinned myocytes, spontaneous Ca\(^{2+}\)/H1\(^{+}\) spark frequency as well as the SR-Ca\(^{2+}\) content were not different between 60-week-old TG and WT mice. However, the Ca\(^{2+}\) spark amplitude was increased (Figure 7d and 7e).

Interestingly, \(I_{\text{NCX}}\) (normalized to the Ca\(^{2+}\) transient and \(C_m\)) was decreased by 40% in cardiomyocytes isolated from older TG mice when compared to age-matched WT (Figure 8a through 8c). In contrast to the group of young TG mice, Ca\(^{2+}\) transport rate was also reduced by 40% in myocytes from 60-week-old TG mice (Figure 8c). Importantly, there was no difference in cardiac NCX1 mRNA expression between old TG mice and aged-matched WT mice. However, a comparison of NCX expression between young and older TG mice suggests that aging is accompanied by downregulation of NCX gene expression in TG mice (Figure 8d versus Figure 4d). This decreased age-dependent NCX gene expression in TG animals is not observed in WT mice and effectively normalizes NCX expression levels in old TG mice to levels measured in WT animals.

**Discussion**

During the development of myocardial diseases, variable shifts in Ca\(^{2+}\) signaling have been reported in stressed cardiomyocytes.\(^\text{14–16}\) This adaptive response depends on multiple factors, many of which are not yet known.\(^\text{21–24}\) It is generally accepted, however, that altered function of RyRs and CICR, RyR-mediated Ca\(^{2+}\) leak, deficient EC coupling, and/or changes in NCX and SERCA2 expression and activity can contribute to impaired myocyte contractility.\(^\text{11,14,15,24}\) To gain insight into these complex interactions, we undertook a comprehensive investigation of cardiomyocyte Ca\(^{2+}\) signaling at 2 different time points during the development of cardiac hypertrophy and failure.\(^\text{10}\) For this purpose, we used transgenic TG1306/1R mice, demonstrating Ang II–mediated cardiac hypertrophy and a gradual transition to heart failure.\(^\text{4}\) Chronic elevation of cardiac Ang II in TG mice induces cardiac hypertrophy in the absence of high blood pressure. The hypertrophy that is observed in young animals evolves into heart failure with aging. Ultimately, the heart of TG animals is characterized by decreased contractility, slowed relaxation, and arrhythmias.\(^\text{4,25}\) In our study, ECC changes observed during the early stages of Ang II–induced cardiac growth may be interpreted as compensatory or adaptive,
whereas maladaptive ECC remodeling was seen to predominate in the more advanced stages of cardiomyocyte and cardiac dysfunction. We propose that a shift in the ratio of SR-Ca\textsuperscript{2+}/H\textsubscript{1001} removal and reuptake (resulting from reduced SERCA2a and NCX expression) is primarily responsible for myocyte dysfunction in older TG cardiomyocytes. Our findings also indicate that higher Ca\textsuperscript{2+} sensitivity of the SR-Ca\textsuperscript{2+}/H\textsubscript{1001} release mechanism may partly compensate for the diminished SR-Ca\textsuperscript{2+} reuptake and content. Our data emphasize the importance of aging and disease progression on ECC dysfunction and subsequent functional decompensation at the organ level.

Compensatory ECC Remodeling in Young TG Myocytes

Our findings show that, at the cellular level, central elements of ECC, such as gain (Ca\textsuperscript{2+}/H\textsubscript{1001} transient to current ratio) and NCX-mediated Ca\textsuperscript{2+} transport, are conserved in young TG myocytes. ECC is very robust with a high degree of “safety,” and therefore it is not surprising that various compensatory mechanisms, including sensitivity of SR-Ca\textsuperscript{2+} release and upregulation of NCX expression, are recruited to preserve ECC even under pathophysiological conditions.

This study confirms our previous findings and shows that SERCA2a expression level is reduced in 20-week-old TG mice (Figure 2).\textsuperscript{4} In intact active cells, the SR-Ca\textsuperscript{2+} content is primarily determined by the SERCA2a activity and I\textsubscript{Ca-L}. It is thus expected that a reduced functional expression of SERCA2a would lead to slower SR-Ca\textsuperscript{2+} reuptake and to lower end-diastolic SR-Ca\textsuperscript{2+} content, associated with reduced ECC gain.\textsuperscript{11} To maintain macroscopic ECC gain and function, Ca\textsuperscript{2+} sensitivity of the SR-Ca\textsuperscript{2+} release may have increased in TG mice to compensate for a reduction in SR-Ca\textsuperscript{2+} content.\textsuperscript{26} Interestingly, chronically elevated cardiac Ang II levels have been reported to accelerate the onset of ECC remodeling that normally occurs during aging via generation of reactive oxygen species.\textsuperscript{27}

In contrast, in skinned myocytes under steady resting Ca\textsuperscript{2+} conditions the SR-Ca\textsuperscript{2+}/H\textsubscript{1001} content is mainly balanced by the open probability of RyRs (ie, the SR-Ca\textsuperscript{2+} leak), the intra-SR-Ca\textsuperscript{2+} buffer conditions, and less by the rate of SR refilling by the SERCA2a.\textsuperscript{28} If the SR-Ca\textsuperscript{2+} leak is not changed appreciably, the reduced functional expression of SERCA2a may not have dramatic consequences for the SR-Ca\textsuperscript{2+} content in the steady state (as is the state seen in Figure 2f). The appearance of spontaneous Ca\textsuperscript{2+} sparks is an indirect measure of the SR-Ca\textsuperscript{2+} leak. Indeed, spontaneous Ca\textsuperscript{2+} release events were found to be unaffected during the development of the cardiac disease phenotype in young TG myocytes, suggesting that defects of RyR function may not contribute to the Ang II–mediated phenotype.

In young TG myocytes, we observed increased I\textsubscript{NCX} density and upregulation of NCX mRNA levels. It could be expected that alterations of NCX expression and/or faster ion cycling (eg, by allosteric NCX regulation) would inevitably result in a concomitant change of NCX current and Ca\textsuperscript{2+} transport rate. However, in TG myocytes increased I\textsubscript{NCX} density and NCX expression levels do not cause any change
in Ca\(^{2+}\) transport rate. We hypothesize that this discrepancy could be explained by variations in cell morphology observed in hypertrophic TG cardiomyocytes. Among cells of different sizes, \(I_{\text{NCX}}\) can only be directly compared with the Ca\(^{2+}\)/H\(_{11001}\) transport when the surface-to-volume relationship remains unchanged. The reason is that \(I_{\text{NCX}}\) changes with the surface of the cell, whereas the rate of NCX dependent decay of free [Ca\(^{2+}\)]\(_i\) is “intrinsically” cell volume–dependent.\(^{29}\) Indeed, diminished surface-to-volume ratio was observed in cardiomyocytes of young TG hearts, and this may explain why Ca\(^{2+}\) transport rate and \(I_{\text{NCX}}\) density are not modified in parallel. Correcting \(I_{\text{NCX}}\) for the cell volume, normalized \(I_{\text{NCX}}\) remained essentially unchanged. Based on the maintained Ca\(^{2+}\) transport rate, we reason that the upregulation of \(I_{\text{NCX}}\) via increased functional expression of NCX molecules occurred as a myocyte effort to compensate for cellular enlargement; that is, an upregulated \(I_{\text{NCX}}\) moves the same amount of Ca\(^{2+}\) per cell volume. Thus, the early upregulation of the NCX is possibly an adaptive process, which later has secondary and undesirable consequences.

### Maladaptive ECC Remodeling in Older TG Myocytes

As previously reported, the older TG phenotype is associated with arrhythmias, changes in potassium \(I_{K1}\) currents, long QT syndrome, and decreases in inotropy, all hallmarks of progression to heart failure.\(^{4,17}\) The Ca\(^{2+}\) signaling profile in the 60-week TG myocytes is distinctive, with maladaptive responses prevailing over adaptive changes. In the older myocyte Ca\(^{2+}\) signaling phenotype, the ratio between sarcolemmal NCX-dependent Ca\(^{2+}\) removal and the SERCA2a-mediated SR-Ca\(^{2+}\) reuptake apparently shifts toward a new steady state. This situation is characterized by a reduced SR-Ca\(^{2+}\) content paralleled by reduced and slowed SR-Ca\(^{2+}\) reuptake. This is supported again by the finding of an accelerated age-dependent decreased functional expression of the SERCA2a.\(^{4}\) In such a dynamic scenario, a secondary downregulation of NCX function is expected to protect SR-Ca\(^{2+}\) content, a finding that was indeed confirmed in older TG cardiomyocytes. However, the NCX downregulation does not completely offset the SR loading effect of SERCA2a activity loss. A new steady state would be achieved very gradually by a beat to beat transition, otherwise rapid net loss of SR-Ca\(^{2+}\) would have fatal consequences. At this later stage, a reduced SR-Ca\(^{2+}\) content could also be partly beneficial, because an increased SR-Ca\(^{2+}\) load might be expected to have undesirable arrhythmogenic side effects. Thus, the reduced SR-Ca\(^{2+}\) content may allow maintenance of myocyte contraction for an extended period, albeit in the context of a smaller global systolic Ca\(^{2+}\) transient.

The finding that even under conditions of low SR-Ca\(^{2+}\) content the ECC gain remained nearly unchanged was surprising. To explain this finding, we hypothesize that the SR-Ca\(^{2+}\) release may be more sensitive to the Ca\(^{2+}\) trigger, suggesting Ca\(^{2+}\) “hypersensitivity” of RyRs.\(^{30,31}\) Supporting evidence for sensitized RyRs comes from the observation of increased Ca\(^{2+}\) spark amplitude. However, our data do not exclude the possibility that other targets in addition to the RyRs contribute to the maintained ECC gain in the Ang II–induced hypertrophic phenotype. RyR functional alteration...
may involve several mechanisms including reactive oxygen species modification or change in the phosphorylation state. The elevated Ca\(^{2+}\) sensitivity of the RyRs and of the ECC mechanism may constitute an important mechanism which compensates for the reduced SR-Ca\(^{2+}\) content to maintain myocyte contraction even with heart failure progression. Over the long term, the deficit in the global systolic Ca\(^{2+}\) transient based on progressively reduced SR-Ca\(^{2+}\) content cannot be adequately compensated for by adaptation changes in Ca\(^{2+}\) handling, leading to a final ECC maladaptive remodeling and demise. Further investigation of Ca\(^{2+}\) fluxes and transients in the working myocyte/myocardium will assist in developing an understanding of how Ca\(^{2+}\) handling is altered under loaded conditions during the transition from adaptation to maladaptation.

Significance and Limitations of the Model

Management of heart failure with left ventricular hypertrophy and diastolic and/or systolic dysfunction in humans is achieved by the use of different agents including angiotensin-converting enzyme inhibitors and AT\(_1\) receptor blockers. The benefit of renin–angiotensin inhibition in heart failure has been demonstrated in several randomized clinical trials. We have previously shown that the hypertrophy in the TG mice can be reversed by pharmacological suppression of the RAS, and future studies are required to determine whether reversal of ECC remodeling can be achieved by these means. The question of whether ECC adaptation/maladaptation and cardiomyocyte hypertrophy are separate or interdependent phenomena arises and cannot be directly resolved by the present findings. Distinct "pathological" (Goq signaling mediated) and "physiological" (phosphatidylinositol 3-kinase/Akt signaling–mediated) hypertrophic states have been characterized, and there is evidence of complex cross talk between these 2 signaling pathways. Although a systematic understanding of ECC adaptation patterns associated with different hypertrophic conditions is yet to emerge, altered Ca\(^{2+}\) homeostasis is a central, albeit variable, phenotype.

The TG mouse model offers a useful and clinically relevant model of hypertrophic cardiomyopathy, recapitulating a common clinical scenario where concentric, compensated hypertrophy ultimately decompensates with dilation and transition to arrhythmogenic failure associated with premature mortality. The temporal pattern of altered Ca\(^{2+}\) handling described in this study may be distinctive of an Ang II–Goq pathology in the nonhypertensive setting. Cardiac Ang II levels increase in conditions of volume overload and in the postinfarct heart. A correlation between endogenous Ang II levels and heart size has been observed in young normotensive, suggesting a link between chronic elevation of local Ang II production and cardiac pathophysiologically. However, one must keep in mind that the present data were obtained from isolated cells and that Ca\(^{2+}\) signaling in vivo is also modulated by many factors, such as mechanical loading conditions and neurohumoral signals. In the in vivo situation, cardiomyocyte hypertrophy, senescence and cell death, fibroblast proliferation, and increased fibrosis produce heterogeneity in electric conduction. Ang II is also reported to modify gap junction properties and to alter cell-to-cell communication in the damaged heart. Finally, Ang II can induce ion channel remodeling and change Ca\(^{2+}\) influx. Blockade of the RAS may, therefore, offer therapeutic benefits against electric remodeling. Experimental antiarrhythmic agents for atrial and ventricular fibrillation include angiotensin-converting enzyme inhibitors and angiotensin type I receptor blockers. In this regard, the present study supports an important role for Ang II in the regulation of Ca\(^{2+}\) handling protein expression and activity and suggests that enhancement of cardiac contractility might be achieved by modulating cardiomyocyte Ang II signaling.

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Disclosures

None.

References


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Angiotensin II–Mediated Adaptive and Maladaptive Remodeling of Cardiomyocyte Excitation–Contraction Coupling: Correction

In an article that appears on page 42 of the July 2, 2009, issue, in Figure 2, the second row, left panel, the integral for the NCX current was mistakenly omitted. Also in Figure 2, the second row, panel C, below the last two bar graphs, the integral for the NCX current (in brackets) was mistakenly omitted. These omissions have been corrected in the figure that is printed below. The authors regret the error.

This error has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/105/1/42

Reference


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Transgenic animals, cell isolation. In brief, mice were heparinized (50 U ip), killed by cervical dislocation and the excised hearts were mounted on a Langendorff perfusion system. After perfusion with nominally Ca\(^{2+}\)-free Tyrode’s solution for 3-5 minutes, at 37°C, collagenase II (14 U/ml, Worthington type 2), protease (0.2 U/ml, Sigma, type XIV). After additional 6-9 minutes of perfusion, hearts were removed from the Langendorff apparatus and the ventricles were cut in small pieces, followed by gentle trituration to obtain a cell suspension. Subsequently, the Ca\(^{2+}\) concentration was slowly raised (over 60 minutes) to 1.8 mmol/L [Ca\(^{2+}\)]\(_o\).

Flash-photolysis in combination with confocal microscopy. The Ca\(^{2+}\) imaging set-up was based on a confocal laser-scanning microscope (MRC 1000 or µRadiance, Bio-Rad, Hemel Hemstead, U.K.; excitation at 488 nm with 50 μW, fluo-3 emission wavelengths > 515 nm) working in the line-scan mode (500 lines/s) combined with a xenon short-arc flash lamp (Strobex 238/278, 230 Ws, pulse duration ≈1ms) to photolyze intracellular DM-nitrophen in an epi-illumination arrangement.¹ For NCX functional studies myocytes were treated with 10 μmol/L ryanodine and 0.5 μmol/L thapsigargin (both from Alomone Labs., Israel) at room temperature (40 min, 20-24°C). Changes of the extracellular solutions were performed using a gravity driven superfusion system (t\(_{1/2}\)= 400 ms, 1 ml/min). The superfusion solution contained (mmol/L): NaCl(140), KCl(5), CaCl\(_2\)(1.8), CsCl(1), BaCl\(_2\)(0.5), HEPES(10), glucose(10), pH 7.4
(NaOH). The pipette filling solution contained (mmol/L): Cs-aspartate (120), tetraethylammonium-Cl₂, (20, Sigma), Na₄-DM-nitrophen (4, Calbiochem, USA), K₅-fluo-3 (0.1, TEFLABS, USA), CaCl₂(0.5), reduced glutathione (GSH, 2), K₂-ATP(5), Hepes(20); pH 7.2 (CsOH). Mg²⁺ was omitted in the flash photolysis experiments, but otherwise MgCl₂ (1.1 mmol/L) was generally present. All experiments were performed at room temperature (20–23°C).

**Voltage clamp and data analysis.** The $I_{\text{NCX}}$, $I_{\text{Ca-L}}$ and the membrane capacitance ($C_m$) measurements were recorded in the whole-cell configuration using patch-clamp technique as previously described. In some cells the myocyte volume was obtained together with $C_m$. Stacks of confocal fluorescence images were recorded and cell volume calculated as previously described by Satoh *et al.*, 1996.² In intact myocytes the SR-Ca²⁺ content is measured after emptying the SR with caffeine and subsequently applying a loading protocol. A train of Ca²⁺ currents in a defined time window was used to ensure comparable [Ca²⁺] influx to reload the SR. In intact cells the SR-Ca²⁺ content is thus mainly determined by the balance of the open probability of the RyRs (the Ca²⁺ leak), the intra- SR-Ca²⁺ buffer conditions and the activity of the SR-Ca²⁺ ATPase (SERCA2).

**Ca²⁺ spark measurements.** Isolated myocytes were permeabilized by exposure to saponin (0.005%, 30-40 sec) in the recording solution.³ The frequency and spatio-temporal parameters of the Ca²⁺
sparks were determined from the line scan images. Solution contained (in mmol/L): K-Asp(120), K$_2$-ATP(3), EGTA-KOH(1), phosphocreatine(10), creatine phosphokinase (5 U/ml), reduced L-glutathione (5), dextran (4%, M.W. 40'000), K$_5$fluor-3 (0.05), HEPES(10), pH 7.2. The free [Ca$^{2+}$]$_i$ was adjusted to 50 nmol/L (add 0.2167 mmol/L CaCl$_2$) and the free [Mg$^{2+}$]$_i$ to 1 mmol/L (add 3.272 mmol/L MgCl$_2$) based on calculations by using WebMAX 10/06: (http://www.stanford.edu/~cpatton/maxc.html). In permeabilized cells there is an infinite ‘cytosolic’ volume and steady resting Ca$^{2+}$ conditions ([Ca$^{2+}$]$_{cyt}$ is ‘clamped’). Over time (cells spend several minutes in the ‘intracellular’ solution) the SR-Ca$^{2+}$ content is largely independent of the rates of SR refilling and number of active SERCA2, but is potentially modulated by SR-Ca$^{2+}$ leak and intra-SR Ca$^{2+}$ buffering.

**Cardiac protein extraction and Western blotting.** Protein expression was measured by Western blotting on hearts from TG and WT littermates. Cardiac tissue was homogenized in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors (cOmplete, and PhosSTOP, Roche) according to recommended brand protocols. For immunodetection of SERCA2a (N-19, SantaCruz Biotechnologies), nitrocellulose membranes were incubated with diluted primary antibodies (1:1000) for 2 hours at room temperature. After 3 series of washing (1xPBS in 1% skim milk), blots were incubated with peroxidase-conjugated (HRP) secondary antibodies and bands were revealed using the ECL
chemiluminescence detection system (PerkinElmer Life Sciences Inc) on autoradiography film (Kodak Biomax MR Film). Autoradiographic blots were scanned (Epson Stylus Photo RX600) and semi-quantitatively analyzed by densitometry using Photoshop CS3. Values were expressed (in arbitrary units) as SERCA2A protein levels detected and normalized to the housekeeping protein GAPDH (ab9483, Abcam). No age- or genotype-dependent variations in GAPDH expression levels were observed.

**Cardiac RNA extraction and real-time PCR.** mRNA expression levels were assessed by real-time PCR using homogenate extract from hearts of TG1306 and WT littermate mice. Total RNA was purified from heart tissue by Trizol reagent (Invitrogen), according to manufacturer’s protocol. RNA samples were subsequently analyzed and quantified by spectrophotometry using a Nanodrop ND-100 UV-Vis Spectrophotometer (Nanodrop Technologies). As a further check of RNA quality and quantity, each RNA sample was electrophoresed using a microfluidic Bioanalyzer 2100 (Agilent Technologies). Results were visualized for expression of and integrity of 21S and 18S rRNA bands. First-strand synthesis of cDNA from purified RNAs (2mg/20ml) was performed using oligo(dT)15 primers (10 mmol/L, Promega) and the reverse transcription Omniscript kit (Qiagen) according to manufacturer’s protocol. Resulting cDNAs were subjected to quantitative real-time Polymerase Chain Reaction (qRT-PCR) using the TaqMan 7500 Fast Real-Time PCR System apparatus (Applied Biosystems) and TaqMan Chemistry that uses fluorogenic probes (TaqMan MGB probes, FAM dye-labelled) to enable the detection of specific
PCR products as they accumulate during PCR. Two distinct TaqMan Gene Expression Assays, containing a specific probe and primer pairs, were ordered for the amplification of the NCX1 (Slc8a1): Mm01232249_m1 (Exon boundary 2-3), Mm00441524_m1 (Exon boundary 10-11). Analysis for relative quantification of gene expression was performed using the comparative C_{T} method (DDC_{T}), which extrapolates starting cDNA levels using the time-dependent log-linear amplification phase of each reaction. Data reported in the Result section were calculated as a gene of interest-to-GAPDH ratio (in arbitrary units). Data obtained for each gene expression assay were combined and averaged. Results are expressed as ‘%-change’ of the gene of interest in TG1306 compared to WT hearts. Values were expressed as means±SEM, 1-way ANOVA t test was used to test for significance.

**Figure 3 (additional details):** The fluorescence was normalized and expressed as F/F_{0}, where F_{0} is the baseline fluorescence at the beginning of each recording. Amplitude and time course of Ca^{2+} concentrations were computed off-line from line-scan images using a customized version of the NIH Image software (NIH, USA) and IDL software (Research Systems). The [Ca^{2+}]_{i} was calculated using a self-ratio method assuming a resting [Ca^{2+}]_{i} of 100 nmol/L and a dissociation constant of the fluo-3:Ca^{2+} complex (K_{d}) of 400 nmol/L.\textsuperscript{6}

The I_{NCX} activation via photorelease of “caged” Ca^{2+} is the methodology of choice and offers the advantage that the substrates for the ion exchange process mediated by the NCX can be controlled
nearly perfectly on both sides of the membrane. In this respect, the \([\text{Ca}^{2+}]_i\) transient amplitude can be adjusted and depends on the UV-flash intensity while \([\text{Ca}^{2+}]_o\) and \([\text{Na}^+]_o\) are controlled by the composition of the superfusion solution. Furthermore, no voltage steps which could induce other contaminating ion currents are required to activate \(I_{\text{NCX}}\). Because of competing \(\text{Ca}^{2+}\) transport mechanisms the SR as a sink or source for \(\text{Ca}^{2+}\) has to be eliminated to establish NCX function and its \(\text{Ca}^{2+}\)-dependence. This is shown in figure 3b where the \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release (CICR) was blocked in the presence of thapsigargin and ryanodine. \(I_{\text{NCX}}\) activated by flash-photolytic \([\text{Ca}^{2+}]_i\)-jumps were sensitive to 6 mmol/L extracellular \(\text{Ni}^{2+}\), suggesting that the observed inward currents are exclusively mediated by the NCX (Fig.3c). For statistical comparison we normalized the \(I_{\text{NCX}}\) density to the \(\text{Ca}^{2+}\) transient amplitude - accounting for variability of the intracellular \(\text{Ca}^{2+}\)-jumps induced by flash-photolysis.
**Online Table I**

Qualitative genotype comparison of ECC parameters obtained in age matched groups

<table>
<thead>
<tr>
<th></th>
<th>Young TG vs. WT</th>
<th>Older TG vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_m$</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Myocyte volume</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Surface/volume ratio</td>
<td>↓</td>
<td>~</td>
</tr>
<tr>
<td>$I_{Ca-L}$</td>
<td>↑</td>
<td>~</td>
</tr>
<tr>
<td>ECC-gain</td>
<td>↓</td>
<td>~</td>
</tr>
<tr>
<td>SR-Ca$^{2+}$ content (whole cell)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Spontaneous Ca$^{2+}$ spark frequency</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>Ca$^{2+}$ spark amplitude</td>
<td>~</td>
<td>↓</td>
</tr>
<tr>
<td>SR-Ca$^{2+}$ content (permeabilized cells)</td>
<td>~</td>
<td>↓</td>
</tr>
<tr>
<td>Peak $I_{NCX}$</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>NCX mediated Ca$^{2+}$ transport</td>
<td>~</td>
<td>↓</td>
</tr>
<tr>
<td>NCX1 mRNA</td>
<td>↑</td>
<td>~</td>
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<tr>
<td>SERCA2 protein$^5$</td>
<td>↓</td>
<td>~</td>
</tr>
</tbody>
</table>

↑, significant increase; ↓, significant decrease; ~, no significant change; small arrow (↓↑), trend
Online Table II
Quantitative comparison of cellular and ECC parameters obtained in age matched groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young WT</th>
<th>Young TG</th>
<th>Old WT</th>
<th>Old TG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular characteristics and ECC gain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell membrane capacitance, $C_{m}$ (pC)</td>
<td>$120.3\pm15.9$, n=10</td>
<td>$155.4\pm14.8$, n=10$^*$</td>
<td>$133.5\pm11$, n=6</td>
<td>$227.4\pm26$, n=6$^{**}$</td>
</tr>
<tr>
<td>Myocyte volume (pl)</td>
<td>$22.3\pm7.4$, n=12</td>
<td>$35.2\pm4.3$, n=10$^*$</td>
<td>$37.5\pm3.8$, n=10</td>
<td>$47.2\pm3.3$, n=10$^*$</td>
</tr>
<tr>
<td>Surface-to-volume ratio</td>
<td>$5.4\pm0.7$, n=10</td>
<td>$4.4\pm0.4$, n=10$^*$</td>
<td>$5.6\pm0.9$, n=12</td>
<td>$5.2\pm0.5$, n=12</td>
</tr>
<tr>
<td><strong>SR Ca$^{2+}$ content (Caffeine)</strong></td>
<td></td>
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<tr>
<td>$I_{\text{INCX}}$ (pA)</td>
<td>$89.2\pm15.3$, n=4</td>
<td>$65.3\pm12$, n=4</td>
<td>$55\pm6.4$, n=6</td>
<td>$40.3\pm5.7$, n=6</td>
</tr>
<tr>
<td>Ca$^{2+}$ transient, $F/F_0$</td>
<td>$5.5\pm0.7$, n=4</td>
<td>$3.8\pm0.5$, n=4</td>
<td>$4.6\pm0.58$, n=6</td>
<td>$3.2\pm4.1$, n=6</td>
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<tr>
<td>SR Ca$^{2+}$ content (pC)</td>
<td>$120.7\pm7.3$, n=4</td>
<td>$97.2\pm6.1$, n=4</td>
<td>$85\pm5.2$, n=6</td>
<td>$48.5\pm5.7$, n=6</td>
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<tr>
<td><strong>Spontaneous Ca$^{2+}$ release (skinned myocytes)</strong></td>
<td></td>
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<tr>
<td>Ca$^{2+}$ spark frequency (s$^{-1}$μm$^{-1}$)</td>
<td>$9.8\pm0.4$, n=6</td>
<td>$10.5\pm1.1$, n=6</td>
<td>$9.75\pm0.53$, n=6</td>
<td>$8.63\pm0.67$, n=6</td>
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<tr>
<td>Ca$^{2+}$ spark amplitude, $F/F_0$</td>
<td>$1.89\pm0.058$, n=6</td>
<td>$2.05\pm0.12$, n=6</td>
<td>$1.85\pm0.015$, n=6</td>
<td>$1.96\pm0.028$, n=6</td>
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<tr>
<td>$I_{\text{INCX}}$ induced by UV-flash photolysis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$I_{\text{INCX}}$ density, $I_{\text{INCX}}/(C_m [\text{Ca}^{2+}])$, (10$^{-3}$ pA/(pF nM))</td>
<td>$0.83\pm0.36$, n=10</td>
<td>$1.46\pm0.29$, n=15</td>
<td>$2.89\pm0.76$, n=7</td>
<td>$1.75\pm0.38$, n=8</td>
</tr>
<tr>
<td>Ca$^{2+}$ transport rate, $\langle \partial [\text{Ca}^{2+}] / \partial t \rangle$, (nMms$^{-1}$)</td>
<td>$0.42\pm0.1$, n=10</td>
<td>$0.56\pm0.15$, n=15</td>
<td>$0.78\pm0.25$, n=7</td>
<td>$0.52\pm0.18$, n=8</td>
</tr>
</tbody>
</table>
REFERENCES:


