Angiotensin II–Mediated Phenotypic Cardiomyocyte Remodeling Leads to Age-Dependent Cardiac Dysfunction and Failure

Andrea A. Domenighetti, Qing Wang, Marcel Egger, Stephen M. Richards, Thierry Pedrazzini, Lea M.D. Delbridge

Abstract—Chronic elevation of plasma angiotensin II (Ang II) is detrimental to the heart. In addition to its hemodynamic effects, Ang II exerts cardiotropic actions that contribute to cardiomyocyte remodeling. However, it remains to be clarified whether these direct actions of Ang II are sufficient to cause contractile dysfunction and heart failure in the absence of altered hemodynamic conditions. In this study, we used TG1306/1R (TG) mice that develop Ang II–mediated cardiac hypertrophy in absence of elevated blood pressure to investigate the phenotypic changes in cardiomyocytes during the adaptive response to chronic cardiac-specific endogenous Ang II stimulation. A 94-week longitudinal study demonstrated that TG mice develop dilated cardiomyopathy with aging and exhibit a significant increase in mortality compared with wild-type (WT) mice. Cardiac hypertrophy in TG mice is associated with cardiomyocyte hypertrophy (15 to 20 weeks: length +20%; 35 to 40 weeks: length +10%, width +15%) but not collagen deposition. In vivo analysis of cardiac function revealed age-dependent systolic and diastolic dysfunction in TG mice (=45% reduction in dP/dtmax and dP/dtmin at 50 to 60 weeks of age compared with WT). Analysis of isolated cardiomyocyte isotonic shortening showed impaired contractility in TG cardiomyocytes (30% to 40% decrease in rates of shortening and lengthening). In TG hearts, chronic Ang II exposure induced downregulation of the sarcoplasmic reticulum calcium pump (SERCA2) and diminution of Ca2+ transients, indicative of an underlying disturbance in calcium homeostasis. In conclusion, chronic Ang II myocardial stimulation without hemodynamic overload is sufficient to produce cardiomyocyte and cardiac dysfunction culminating in heart failure. (Hypertension. 2005;46:426-432.)

Key Words: aging ■ cardiac function ■ heart failure ■ hypertrophy ■ myocytes ■ renin-angiotensin system

The renin-angiotensin system (RAS) plays a crucial role in cardiovascular regulation via the activity of its effector, angiotensin II (Ang II), which is a potent vasoconstrictor and modulator of fluid balance. Ang II also exerts direct trophic actions on cardiac cells, inducing cardiomyocyte hypertrophy and fibroblast proliferation.

Local Ang II production is of key importance in the pathophysiology of the RAS in the heart.2 Gradual increases in cardiac Ang II levels have been reported in experimental models and clinically during the development of heart failure.3,4 Numerous studies have demonstrated the efficacy of RAS blockade in the treatment of cardiac remodeling and heart failure, independently of the reduction in systemic blood pressure.5,6 Elevated cardiac angiotensinogen levels are observed in various animal models of pressure and volume overload cardiac hypertrophy, in which Ang II is considered to contribute significantly to cardiac remodeling through its growth-promoting properties.7,8

In addition, Ang II is an important modulator of cardiac and cardiomyocyte contractility. Acute in vitro and in vivo exposure of the myocardium to Ang II usually (but not always) increases contractility.9–12 In contrast, Ang II has been shown to exacerbate contractile dysfunction in experimental models of pressure-overload cardiac hypertrophy13 and pacing- or infarction-induced heart failure.14,15

Little is known about the chronic effects of high levels of intracardiac Ang II on cardiac function. In particular, the hemodynamics-independent impact of chronic Ang II overproduction in the heart on cardiomyocyte contractility has yet to be evaluated. The present study investigates the TG1306/1R (TG) mouse model, which exhibits cardiac-specific elevation of Ang II production.16,17 In the TG, which has been shown previously to develop Ang II–mediated cardiac hypertrophy in the absence of elevated blood pressure, we evaluate the direct effects of endogenous cardiac Ang II overproduction on cardiomyocyte and heart morphology and function.
Methods

Methods are detailed in the online supplement, available at http://www.hypertensionaha.org.

Experimental Model

We reported previously the generation of a transgenic heterozygous mouse, the normotensive and hypertrophic TG, which carries multiple copies of the rat angiotensinogen gene under control of the α-myosin heavy chain promoter, and exhibits significantly elevated angiotensinogen expression from early development through to senescence. Experiments were conducted on male mice 15 to 20, 25 to 30, 35 to 40, and 50 to 60 weeks of age. Longevity data were also collected for mice over a period of 94 weeks.

Cardiac Function, Heart Histology, and Cardiomyocyte Morphometry

Whole heart contractility in conscious mice was determined by the measurement of left ventricular (LV) pressure derivative dP/dt as described previously using a polyether block amide (Pebax) catheter. For histological analysis, transverse sections were stained (Van Gieson’s) and analyzed for collagen content by computer-assisted densitometric morphometry (collagen expressed as percentage of total sectional area).

Cardiomyocyte Contractility and Ca2+ Measurements

Isolated ventricular cardiomyocytes were prepared by collagenase retrograde aortic perfusion methods described previously. Cell dimensions were measured under bright-field microscopy (100 cardiomyocytes per heart). Cardiomyocyte contractility was evaluated using a rapid imaging system, as described previously. Cells were paced using a frequency ramp (1.5, 3.0, 4.0, and 5.0 Hz). For each contraction cycle, a range of normalized contractile parameters was automatically computed and averaged (see Table 3 legend).

Caffeine-induced Ca2+ transients were evaluated in cardiomyocytes isolated from 25- to 30-week-old mice by fluo-3 fluorescence measurements using confocal laser-scanning microscopy as described previously. Using stimulation conditions to ensure comparable sarcoplasmic reticulum (SR) Ca2+ loading conditions, Ca2+ transient kinetics after application of caffeine were evaluated.

Western Blot Analysis of the SR Ca2+ ATPase

SR Ca2+ ATPase (SERCA2) levels were determined by Western blot from ventricular homogenate protein extract using goat anti-mouse polyclonal antibodies and peroxidase-conjugated secondary anti-goat IgG antibodies. Bands were identified using chemiluminescence.

Statistics

Results are expressed as mean±SEM. One-way and 2-way ANOVAs were applied as indicated to test for differences (P<0.05) between age-matched TG and wild-type (WT) mice.

Results

Survival and the Hypertrophic Phenotype

Compared with WT littermate controls, TG mice exhibited a significant increase in mortality (Figure 1) over the 94-week longitudinal study (WT 83% survival versus TG 46% survival). Postmortem analysis showed that premature mortality in the TG group was predominantly associated with occurrence of a dilated cardiac phenotype, whereas the survivor TG mice exhibited a concentric hypertrophic phenotype (Figure 2A through 2C). To understand whether remodeling severity and lethality were associated with variable cardiac Ang II production levels, we generated a small number of homozygous TG mice harboring double the transgene complement.

Depressed Cardiac Function In Vivo Associated With Cardiomyocyte Remodeling

To determine whether hypertrophic remodeling was associated with cardiac dysfunction in vivo, we assessed LV contractility of 15- to 20-week-old and 50- to 60-week-old TG mice by intraventricular catheterization (Table 2). In the younger TG mice, early evidence of relaxation abnormality was detected as a significant increase in the time constant of isovolumic pressure decline (tau). With increased maturity, more extensive signs of systolic and diastolic dysfunction...
were observed, with an \(-45\%\) decrease in rate of LV pressure development \((\frac{dP}{dt_{\text{max}}}}\) and relaxation \((-\frac{dP}{dt_{\text{min}}}}\) accompanying the further deterioration in relaxation time constant in TG mice. Our results confirmed that even at 50 to 60 weeks of age, TG mice remained normotensive despite the development of Ang II–dependent cardiac hypertrophy.16,17

### Cardiomyocyte Contractile Dysfunction in Transgenic Mice

Cardiomyocyte excitation–contraction coupling was assessed in vitro by measurement of isotonic shortening of single cardiomyocytes over a range of pacing frequencies (1.5 to 5.0 Hz). Cells of similar dimension were selected for this comparison (length 129.0 \( \pm \) 13.0 \( \mu \text{m} \) length and width 29.0 \( \pm \) 3.0 \( \mu \text{m} \)) to avoid confounding the data with size-dependent performance factors.

Comparisons between age-matched TG and WT at 5 Hz showed that both rates of shortening and lengthening (maximal rate of cell shortening \([\text{MRS}]\) and maximal rate of cell lengthening \([\text{MRL}]\)) were reduced in the TG myocytes relative to WT controls (Table 3). The slowest contraction kinetics were observed in the group of older TG myocytes. Genotype-dependent prolongation effects on contraction cycle timing were also evident at both ages: the latency \((T_o)\), the shortening and lengthening periods, and total cycle times were significantly longer in TG than WT myocytes. These genotype differences were maintained across the range of pacing frequencies (Figure 3). A negative frequency “staircase” was observed for all parameters at both ages, except for the latency \((T_o)\), which increased with frequency (Figure 3D).

Across the range of pacing frequencies, WT and TG myocytes from older animals exhibited reduced maximal shortening \([\%S]\) and rate of shortening \([\text{MRS}]\), together with an abbreviated duration of shortening time \((T_s-T_e)\) relative to their younger counterparts (Figure 3A, 3B, and 3F). These findings demonstrate that in mouse myocytes, ageing, per se, regardless of genetic type, is associated with blunted and abbreviated shortening activity. Interestingly, neither the rate of lengthening \([\text{MRL}]\) nor cycle time \((T_e-T_s)\) exhibited age-dependent modulation in the TG (Figure 3C and 3E).

### TABLE 1. Age-Dependent Cardiac Structural Remodeling (But Not Fibrosis) in TG Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>15–20 Week WT</th>
<th>15–20 Week TG</th>
<th>35–40 Week WT</th>
<th>35–40 Week TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, mg</td>
<td>131.3 ( \pm ) 2.5 (20)</td>
<td>152.6 ( \pm ) 6.7* (20)</td>
<td>145.1 ( \pm ) 3.6 (20)</td>
<td>200.5 ( \pm ) 9.4* (20)</td>
</tr>
<tr>
<td>CWI, mg/g</td>
<td>4.9 ( \pm ) 0.1 (20)</td>
<td>5.4 ( \pm ) 0.1* (20)</td>
<td>4.5 ( \pm ) 0.1 (20)</td>
<td>5.5 ( \pm ) 0.2* (20)</td>
</tr>
<tr>
<td>LV collagen, %tsa</td>
<td>1.0 ( \pm ) 0.2 (11)</td>
<td>1.1 ( \pm ) 0.3 (11)</td>
<td>1.3 ( \pm ) 0.2 (6)</td>
<td>1.2 ( \pm ) 0.2 (6)</td>
</tr>
<tr>
<td>RV collagen, %tsa</td>
<td>3.1 ( \pm ) 0.3 (11)</td>
<td>3.3 ( \pm ) 0.3 (11)</td>
<td>3.2 ( \pm ) 0.2 (6)</td>
<td>3.5 ( \pm ) 0.2 (6)</td>
</tr>
<tr>
<td>LV lumen area, mm(^2)</td>
<td>1.4 ( \pm ) 0.1 (10)</td>
<td>1.4 ( \pm ) 0.1 (11)</td>
<td>2.3 ( \pm ) 0.2 (6)</td>
<td>2.6 ( \pm ) 0.2 (6)</td>
</tr>
<tr>
<td>LV free wall, mm</td>
<td>1.1 ( \pm ) 0.02 (10)</td>
<td>1.4 ( \pm ) 0.05* (10)</td>
<td>1.1 ( \pm ) 0.05 (6)</td>
<td>1.5 ( \pm ) 0.05* (6)</td>
</tr>
<tr>
<td>Myocyte length, ( \mu \text{m} )</td>
<td>150.9 ( \pm ) 2.5 (7)</td>
<td>178.7 ( \pm ) 3.8* (7)</td>
<td>157.1 ( \pm ) 1.8 (6)</td>
<td>168.4 ( \pm ) 3.9* (6)</td>
</tr>
<tr>
<td>Myocyte width, ( \mu \text{m} )</td>
<td>27.7 ( \pm ) 0.7 (7)</td>
<td>29.0 ( \pm ) 1.0 (7)</td>
<td>27.5 ( \pm ) 0.5 (6)</td>
<td>31.3 ( \pm ) 0.5* (6)</td>
</tr>
</tbody>
</table>

Collagen density expressed as percent of total sectional area occupied by collagen of total field analyzed (%tsa). No. of animals shown in parentheses.

CWI indicates cardiac weight index; RV, right ventricular.

*\(P<0.05\), TG vs age-matched WT.

Figure 2. Transverse sections of hearts from WT and TG. WT 94 weeks and 1 week (A and D); TG heterozygous 1 week (E) and 94 weeks, exhibiting contrasting concentric and dilated phenotypes (B and C); TG homozygous 1 week (F).
SERCA2 Expression and Cardiomyocyte Ca\textsuperscript{2+} Transients

Because contraction kinetics are largely dependent on SR Ca\textsuperscript{2+} homeostasis, we investigated the expression of the SR SERCA2 Ca\textsuperscript{2+} pump in the hearts of 15- to 20-week-old TG and WT mice (Figure 4A and 4B). Western blot analysis showed an ≈80% downregulation of the SERCA2, suggesting that alterations in Ca\textsuperscript{2+} handling are potentially involved in relaxation dysfunction in TG hearts. This is supported by measurements of Ca\textsuperscript{2+} transients in cardiomyocytes of 25- to 30-week-old mice. The amplitude of Ca\textsuperscript{2+} transient in TG myocytes was ≈50% decreased when compared with WT cells (Figure 4C and 4D). In addition, the decay of the caffeine-induced Ca\textsuperscript{2+} transients was prolonged in TG myocytes compared with WT (WT \( \tau = 798 \pm 177 \) ms; TG \( \tau = 1508 \pm 412 \) ms; \( P<0.05 \)).

Discussion

The present study demonstrates that chronic overexpression of cardiac Ang II is sufficient to induce the development of 2 contrasting hypertrophic phenotypes in aged transgenic mice. Those TG animals that died during the 94-week observation period exhibited a dilated hypertrophic phenotype, whereas their longer-surviving TG littermates were characterized by a concentric hypertrophy. These findings indicate that the concentric hypertrophic state is associated with a degree of functional compensation and survival, and that the dilated phenotype emerges in association with increased mortality. Our results also suggest that functional decompensation precedes overt myocardial dilation and support the hypothesis that concentric and dilated hypertrophy share some common cellular pathways of development.\textsuperscript{21}

Our observations in the homozygous TG mice suggest that an increased expression of the angiotensinogen transgene, and therefore of Ang II production, exacerbates the severity of the cardiac phenotype observed, indicating that the type and severity of Ang II–mediated myocardial remodeling are time- and angiotensinogen gene copy–dependent. Our previous studies on TG mice already demonstrated that the trophic actions of Ang II on the heart are mediated by Ang II type 1 (AT1) receptors.\textsuperscript{16,17} We have also shown that the ratio of expression of receptor subtypes (AT1/AT2) in heterozygous and therefore of Ang II production, exacerbates the severity of the cardiac phenotype observed, indicating that the type and severity of Ang II–mediated myocardial remodeling are time- and angiotensinogen gene copy–dependent. Our previous studies on TG mice already demonstrated that the trophic actions of Ang II on the heart are mediated by Ang II type 1 (AT1) receptors.\textsuperscript{16,17} We have also shown that the ratio of expression of receptor subtypes (AT1/AT2) in heterozygous

Our study extends previous observations that chronic overexpression of cardiac Ang II in transgenic mice is sufficient to induce blood pressure–independent cardiac hypertrophy without signs of fibrosis.\textsuperscript{23} Although qualitative changes in collagen content cannot be excluded, our morphological analysis suggests that cardiac remodeling results almost entirely from cardiomyocyte hypertrophy rather than extracellular matrix deposition. As observed previously,\textsuperscript{24,25} collagen concentrations in the LV free wall were substantially lower than that in the right ventricle, but no age- or genotype-specific differences were detected. Of interest is the apparent similarity between the cardiac phenotype described here and that observed in transgenic mice overexpressing the Gaq protein specifically in the heart. The latter animals are characterized by cardiac decompensation in the absence of cardiac fibrosis and pressure overload.\textsuperscript{26,27} Because signaling pathways mediated by AT1 receptors are known to be Gaq linked,\textsuperscript{28} the cardiac remodeling in the TG mice may be an

### TABLE 2. In Vivo Measurement of Cardiac Function in 15- to 20-Week Old and 50- to 60-Week-Old TG and WT Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>15–20 Week</th>
<th>50–60 Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TG</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>TG</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>606±30</td>
<td>551±23</td>
</tr>
<tr>
<td>Peak LVSP, mm Hg</td>
<td>135±3</td>
<td>137±4</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>5.5±0.9</td>
<td>6.5±1.5</td>
</tr>
<tr>
<td>+dP/dt max, mm Hg/s</td>
<td>15 646±1673</td>
<td>12 997±980</td>
</tr>
<tr>
<td>−dP/dt min, mm Hg/s</td>
<td>−11 825±904</td>
<td>−9836±963</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>3.8±0.4</td>
<td>5.7±0.6*</td>
</tr>
</tbody>
</table>

LVSP indicates LV systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt max and −dP/dt min, maximal rates of LV pressure development and relaxation; Tau, time constant of LV isovolumic relaxation.

Each value was averaged from 120 s of sampling time.

\(*P<0.05\); TG vs age-matched WT; \( n = 6 \) per group.

### TABLE 3. Measurement of Isotonic Shortening of Single Adult Cardiomyocytes (5 Hz)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>15–20 Week</th>
<th>35–40 Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>TG</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>TG</td>
</tr>
<tr>
<td>n (myocytes)</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>%S, L/L0</td>
<td>5.9±0.5</td>
<td>5.7±0.3</td>
</tr>
<tr>
<td>MRS, L/s</td>
<td>4.1±0.2</td>
<td>3.6±0.2*</td>
</tr>
<tr>
<td>MRL, L/s</td>
<td>4.2±0.3</td>
<td>2.9±0.1*</td>
</tr>
<tr>
<td>( T_s )</td>
<td>11.5±0.8</td>
<td>13.7±1.1*</td>
</tr>
<tr>
<td>( T_{r-T_s} ) ms</td>
<td>67.9±3.8</td>
<td>85.1±2.7*</td>
</tr>
<tr>
<td>( T_{r-T_s} ) ms</td>
<td>26.0±1.5</td>
<td>31.3±1.6*</td>
</tr>
<tr>
<td>( T_{r-T_s} ) ms</td>
<td>41.9±2.5</td>
<td>53.8±1.9*</td>
</tr>
</tbody>
</table>

Parameters calculated include the maximum cell shortening (%S), expressed as a percentage of initial resting cell length (L\(_0\)); the excitation–contraction coupling latency measured as the time at which the cell commenced shortening after stimulus application (\( T_s \)); the time at %S (\( T_{r-T_s} \)); the time at which the cell length returned to L\(_0\) (\( T_r \)); the MRS and MRL.

\( T_s – T_{r-T_s} \) duration of shortening; \( T_r – T_s \), duration of lengthening.

\(*P<0.05\); TG vs age-matched WT.
outcome of specific activation of cardiomyocyte AT1 Gq-coupled receptors.

The present study demonstrates that long-term overexpression of cardiac Ang II has a detrimental effect on excitation-contraction coupling in the myocardium, even when there is no elevation of afterload. Myocardial remodeling in 50- to 60-week-old transgenics is associated with decreased in vivo contractility and relaxation, which is preceded by a more subtle sign of relaxation delay observed in hearts of 15- to 20-week-old TG mice. These findings indicate that chronic overproduction of Ang II in the heart causes systolic dysfunction and early onset of diastolic dysfunction (prominent signs of heart failure). Myocyte contractility experiments complement the in vivo data and show that impaired relaxation (MRL) and prolonged contractile cycle time (T1-Tc) in younger TG cardiomyocytes precede later emergence of shortening dysfunction (MRS) and increased latency (T1). This would suggest that the hypodynamic performance of TG hearts in vivo reflects fundamental abnormalities in cardiomyocyte excitation-contraction coupling.

Additionally, these data emphasize the important role played by aging in the development of contractile dysfunction. Cardiomyocytes from older TG and WT exhibit reduced shortening and an abbreviated shortening time. In the WT, aging is associated with a reduction in the rate of myocyte lengthening during relaxation, but in the TG, in which this parameter is already dramatically suppressed at 15 to 20 weeks, no further age-dependent decrement is observed. Thus, aging is associated with deterioration of cardiomyocyte kinetics consistent with the development of failure. Increased levels of Ang II in the heart accelerates the onset of relaxation impairment and exacerbates shortening dysfunction and latency.

Alterations in cardiomyocyte and myocardial function in these mice are associated with the marked reduction in the expression of the SR SERCA2 protein, diminished systolic Ca2+ levels, and prolongation of Ca2+ transients (Figure 4). Downregulation of the SERCA2 Ca2+ pump would suppress the reuptake of Ca2+ into the SR delaying myocyte relaxation, reduce the levels of releasable SR Ca2+, and erode systolic function. In general, differences in TG and WT cardiomyocyte contractile parameters were proportionally consistent over the range of pacing frequencies evaluated, indicating that SR loading rather than interval-dependent excitation-contraction coupling.
contraction coupling recovery, is altered in the TG. These results accord with findings in the Gαq-overexpressing transgenic, in which cardiomyocyte dysfunction (decreased rates of shortening and lengthening) is associated with downregulation of SERCA2 and prolonged duration of Ca²⁺ transients. The specific means by which chronic elevation of cardiac Ang II perturbs cardiomyocyte Ca homeostasis remains to be elucidated. Further molecular and electrophysiological investigations are necessary to explore how cellular compensatory mechanisms are recruited to sustain excitation–contraction coupling in the TG cardiomyocytes before the eventual decompensation transition associated with dilated cardiomyopathy and failure.

**Perspectives**

Our study indicates that chronic activation of the RAS in the heart in absence of hemodynamic overload produces cardiomyocyte and cardiac dysfunction. In particular, endogenous overproduction of cardiac Ang II is sufficient to induce the development of an evolving cardiac phenotype ultimately resulting in dilation and heart failure. These phenotypic alterations likely result from a change in gene expression in cardiomyocytes induced by Ang II. In this regard, the TG model provides a valuable experimental tool for elucidating the cellular pathways responsible for the transition from compensated concentric hypertrophy to dilation and cardiac failure.

**Acknowledgments**

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**References**

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Angiotensin II-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure

Short title: ‘Angiotensin II and cardiomyocyte dysfunction’

Andrea A. Domenighetti, Qing Wang, Marcel Egger, Stephen M. Richards, Thierry Pedrazzini, Lea M. D. Delbridge

From the Department of Physiology (A.A.D., L.M.D.D.), University of Melbourne, Australia; the Department of Medicine (Q.W., T.P.), University of Lausanne Medical School, Switzerland; the Department of Physiology (M.E.), University of Bern, Switzerland and the Division of Biochemistry (S.M.R.), University of Tasmania, Australia.

Correspondence to: Dr. Lea M. Durham Delbridge, PhD
Department of Physiology
University of Melbourne,
Parkville Victoria, 3010
Australia
+61-3-8344-5853 phone
+61-3-8344-5897 fax
lmd@unimelb.edu.au


**Expanded Methods**

**Experimental model**

We have previously reported the generation of a transgenic heterozygous mouse, the normotensive and hypertrophic TG1306/1R, which carries multiple copies of the rat angiotensinogen gene under control of the α-myosin heavy chain (αMHC) promoter, and exhibits significantly elevated angiotensinogen expression from early development through to senescence.\textsuperscript{1-3} In vivo experimental procedures were performed on transgenic (TG) mice and their wild-type littermates (WT) anaesthetized by halothane inhalation (1-2 % in O\textsubscript{2}). For other experiments, hearts were excised from mice anaesthetized by intraperitoneal injection of nembutal (70 mg/kg). Experiments as described below were conducted on male mice aged 15-20, 25-30, 35-40 and 50-60 weeks. Longevity data were also collected for an additional group of TG and WT mice over a period of 94 weeks.

**Analysis of cardiac function in conscious mice**

Whole heart contractility in conscious mice was determined by the measurement of left ventricular (LV) pressure derivative dP/dt, as previously described.\textsuperscript{4} Briefly, a polyether block amide (Pebax\textsuperscript{®}) catheter was advanced into the LV via the right carotid artery. Mice recovered for at least 4 hr before signal recording. The catheter was connected to a pressure transducer (World Precision Instruments), and heart rate, LV systolic pressure, and pressure derivatives were recorded for 15 min at a sampling rate of 1000 Hz using the computerized data acquisition system Notocord HEM 3.1 (Notocord, 78290 Croissy-sur-Seine, France).
**Whole heart histology and morphometry**

The degree of cardiac hypertrophy was established by measuring the ratio between the blotted wet heart weight and the body weight (mg/g), i.e. the cardiac weight index (CWI). Excised hearts were fixed in 10 % buffered formalin, dehydrated and embedded in paraffin blocks. Transversely oriented sections (5 µm) cut at the mid point of the heart were evaluated macroscopically (Haematoxylin-Eosine staining) and were analyzed for collagen content (modified Van Gieson’s staining) by computer-assisted densitometric morphometry (Image Pro Plus) using bright field low power (10x) microscopy. The sectional area occupied by stained collagen was computed for each image and expressed as a percentage of total cross sectional area analyzed (% tsa), estimated at ~8 mm² per heart. Areas of perivascular collagen and large blood vessels were excluded from the field of analysis. Chamber morphology was evaluated by measurement of LV lumenal area (mm²), while LV free wall thickness (mm) was digitally measured from wall transects positioned to avoid papillary muscle protrusions (average of 5 measurements per heart).

**Cardiomyocyte isolation and cell dimension measurements**

Isolated ventricular cardiomyocytes were prepared by collagenase dissociation procedures. Excised hearts were perfused retrogradely via the aorta with a HEPES buffer (in mmol/L): NaCl 142, KCl 5.7, KH₂PO₄.H₂O 1.5, MgCl₂.6H₂O 1.7, HEPES free-acid 10, glucose 11.7, taurine 20 (pH 7.4, 100% O₂ gassed, at 36°C), supplemented with (in mg/ml) BSA 1, collagenase type II 0.4 and collagenase type IV 0.1. Dissociated myocytes were filtered and resuspended in fresh HEPES buffer (36 °C). Cell yield was assessed and cell dimensions (length and width) were measured using a calibrated eyepiece graticule at 400x magnification.
using an inverted light microscope. 100 healthy rod-shaped adult cardiomyocytes were randomly selected for measurement per heart.

**Cardiomyocyte contractility**

Isolated cardiomyocyte contractility was evaluated using a rapid imaging system, as previously described. Cells were superfused at 36.0±0.5 °C with pre-oxygenated, buffered solution containing (in mmol/L): NaCl 142, KCl 5.7, KH$_2$PO$_4$.H$_2$O 1.5, MgCl$_2$.6H$_2$O 1.7, HEPES free-acid 10, CaCl$_2$ 2, glucose 11.7, taurine 20 (pH 7.4, 100% O$_2$ gassed). Cardiomyocytes were field stimulated to shorten isotonically to achieve steady-state contraction (10 min) prior to pacing challenge using a standardized ascending ramp of 4 frequencies (1.5, 3.0, 4.0 and 5.0 Hz). For each contraction cycle a range of normalized contractile parameters were automatically computed (see the legend of Table 3 for details). For every myocyte 30 contraction cycles were averaged at each frequency for calculation of parameter means.

**Cardiomyocyte Ca\textsubscript{i} measurements**

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solutions were performed using a gravity driven superfusion system ($t_{1/2} \approx 300$ ms, 1 ml/min). The protocol consisted of a first brief application of caffeine (20 mmol/L) followed by six depolarizations from -40 mV to 0 mV for 200 ms to ensure comparable SR calcium loading conditions and was completed by a second application of caffeine for 3 sec. Fluo-3 was excited at 488 nm with 50 µW intensity and the fluorescence was detected at wavelengths $>515$ nm. Fluorescence changes were analyzed from line-scan images using a customized version of the NIH Image software (NIH, USA).

**Western blot analysis of the sarcoplasmic reticulum calcium ATPase (SERCA2)**

Sixty µg of total protein extracted from ventricular homogenate was separated using a 10% SDS-polyacrylamide gel and transferred to PVDF membrane. Membranes were incubated overnight (4 °C) with diluted goat anti-mouse SERCA2 polyclonal antibodies (SantaCruz Biotechnology, USA). After washing, membranes were incubated with peroxidase-conjugated secondary anti-goat IgG antibodies (SantaCruz Biotechnology, USA) and bands were identified using chemiluminescence (ECL) on autoradiography film. Bands were analyzed by densitometry and values expressed as SERCA2 protein levels detected and normalized by total protein transferred on PVDF membrane (visualized with Ponceau S stain).

**Statistics**

Results are expressed as mean ± SEM. One-way ANOVA was used to test for differences between age-matched TG and WT mice. Two-way ANOVA with repeated measures was applied to evaluate myocyte contractility group differences as a function of genotype, age and pacing. Statistical significance was recognized at $P<0.05$. 


Angiotensin II-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure

Short title: ‘Angiotensin II and cardiomyocyte dysfunction’

Andrea A. Domenighetti, Qing Wang, Marcel Egger, Stephen M. Richards, Thierry Pedrazzini, Lea M. D. Delbridge

From the Department of Physiology (A.A.D., L.M.D.D.), University of Melbourne, Australia; the Department of Medicine (Q.W., T.P.), University of Lausanne Medical School, Switzerland; the Department of Physiology (M.E.), University of Bern, Switzerland and the Division of Biochemistry (S.M.R.), University of Tasmania, Australia.

Correspondence to: Dr. Lea M. Durham Delbridge, PhD
Department of Physiology
University of Melbourne,
Parkville Victoria, 3010
Australia
+61-3-8344-5853 phone
+61-3-8344-5897 fax
lmd@unimelb.edu.au
Expanded Methods

Experimental model

We have previously reported the generation of a transgenic heterozygous mouse, the normotensive and hypertrophic TG1306/1R, which carries multiple copies of the rat angiotensinogen gene under control of the $\alpha$-myosin heavy chain ($\alpha$MHC) promoter, and exhibits significantly elevated angiotensinogen expression from early development through to senescence.$^{1-3}$ In vivo experimental procedures were performed on transgenic (TG) mice and their wild-type littermates (WT) anaesthetized by halothane inhalation (1-2 % in $O_2$). For other experiments, hearts were excised from mice anaesthetized by intraperitoneal injection of nembutal (70 mg/kg). Experiments as described below were conducted on male mice aged 15-20, 25-30, 35-40 and 50-60 weeks. Longevity data were also collected for an additional group of TG and WT mice over a period of 94 weeks.

Analysis of cardiac function in conscious mice

Whole heart contractility in conscious mice was determined by the measurement of left ventricular (LV) pressure derivative $dP/dt$, as previously described.$^4$ Briefly, a polyether block amide (Pebax®) catheter was advanced into the LV via the right carotid artery. Mice recovered for at least 4 hr before signal recording. The catheter was connected to a pressure transducer (World Precision Instruments), and heart rate, LV systolic pressure, and pressure derivatives were recorded for 15 min at a sampling rate of 1000 Hz using the computerized data acquisition system Notocord HEM 3.1 (Notocord, 78290 Croissy-sur-Seine, France).
Whole heart histology and morphometry

The degree of cardiac hypertrophy was established by measuring the ratio between the blotted wet heart weight and the body weight (mg/g), i.e. the cardiac weight index (CWI). Excised hearts were fixed in 10 % buffered formalin, dehydrated and embedded in paraffin blocks. Transversely oriented sections (5 µm) cut at the mid point of the heart were evaluated macroscopically (Haematoxylin-Eosine staining) and were analyzed for collagen content (modified Van Gieson’s staining) by computer-assisted densitometric morphometry (Image Pro Plus) using bright field low power (10x) microscopy. The sectional area occupied by stained collagen was computed for each image and expressed as a percentage of total cross sectional area analyzed (% tsa), estimated at ~8 mm² per heart. Areas of perivascular collagen and large blood vessels were excluded from the field of analysis. Chamber morphology was evaluated by measurement of LV lumenal area (mm²), while LV free wall thickness (mm) was digitally measured from wall transects positioned to avoid papillary muscle protrusions (average of 5 measurements per heart).

Cardiomyocyte isolation and cell dimension measurements

Isolated ventricular cardiomyocytes were prepared by collagenase dissociation procedures. Excised hearts were perfused retrogradely via the aorta with a HEPES buffer (in mmol/L): NaCl 142, KCl 5.7, KH₂PO₄.H₂O 1.5, MgCl₂.6H₂O 1.7, HEPES free-acid 10, glucose 11.7, taurine 20 (pH 7.4, 100% O₂ gassed, at 36°C), supplemented with (in mg/ml) BSA 1, collagenase type II 0.4 and collagenase type IV 0.1. Dissociated myocytes were filtered and resuspended in fresh HEPES buffer (36 °C). Cell yield was assessed and cell dimensions (length and width) were measured using a calibrated eyepiece graticule at 400x magnification.
using an inverted light microscope. 100 healthy rod-shaped adult cardiomyocytes were randomly selected for measurement per heart.

**Cardiomyocyte contractility**

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