Aging-related cardiomyocyte functional decline is sex- and angiotensinII-dependent

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Running head (<50characters, incl. spaces): aging-related cardiomyocyte dysfunction (40characters)

Keywords: angiotensin overexpression, excitation-contraction coupling, hypertrophy, calcium, ageing, arrhythmogenesis, failure

Text word count: 5574 (excl. summary, references, figure legends)
Abstract word count : 248
No. of figures: 7
No. of tables: 0

References : 49
Supplementary Material: Table S1 & S2, Figure S1 & S2

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Introduction

Heart failure is an age-dependent pathological phenomenon, most prevalent in patients >65years old (de Giuli*, et al.* 2005). Age is the primary risk factor for cardiovascular disease with each 5yr increment over 65yrs associated with 22% increase in risk (Larson 1995). Despite this pivotal link between aging and cardiovascular risk, studies investigating heart failure in aged animal models are lacking. Advances in treatment strategies over the past two decades have led to an improvement in heart failure survival. This improvement has been less marked in relation to outcomes for women (Roger*, et al.* 2004), and attests to a sex disparity in the effectiveness of heart failure treatment. The epidemiology and clinical characteristics of heart failure are identified to be sex-dependent (Barsheshet*, et al.* 2012; Regitz-Zagrosek and Lehmkuhl 2005; Seeland and Regitz-Zagrosek 2012) and evidence from meta-analysis of clinical trials suggests that current heart failure therapies are more effective in men than in women (Barsheshet*, et al.* 2012; Rabi*, et al.* 2008; Shekelle*, et al.* 2003). These data indicate important underlying sex differences in the cardiac pathology of aging-associated failure. Experimentally, an age-dependent reduction in cardiomyocyte Ca^{2+} current influx with age has been identified in female but not male rodents (Howlett 2010). Sex differences in cardiomyocyte dysfunction associated with the progression of heart failure have not yet been investigated.

It is well established that the renin-angiotensin system (RAS) is upregulated in heart failure (Unger and Li 2004) and usual treatment regimes include angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) (Ahmed 2003; Durand 2002). Experimental studies have identified that cardiac RAS upregulation appears to play an important role in aging hearts. In addition to exerting systemic effects, local cardiac RAS actions have been well documented and all components of the RAS are expressed in cardiac tissue (Lindpaintner and Ganten 1991). Left ventricular expression of angiotensinogen and ACE is selectively elevated in hearts of aged (non-failing) male rodents despite an age-induced reduction in systemic RAS activity (Heymes*, et al.* 1994). Myocardial expression of angiotensin II (AngII) receptor subtypes AT_1R and AT_2R is also upregulated in aged males, potentially mediating age-related cardiac pathology (Heymes*, et al.* 1998). Experimentally, sex-specific vascular and renal effects of both systemic RAS stimulation and inhibition have been identified (Brown*, et al.* 2012; Maric 2005; Sullivan 2008) but whether similar sex differences exist in the myocardial effects of RAS activation is unknown. Elevation of cardiac RAS activity in aged female hearts would be expected, although this has not yet been demonstrated. An estrogen-response element has been localised to the angiotensinogen promotor,

and in relatively estrogen-suppressed aged females (where gonadal estrogen production is low), this may be instrumental in regulating local tissue AngII production (Feldmer*, et al.* 1991).

A role for locally produced AngII in cardiac functional and structural remodelling has been demonstrated (Dostal and Baker 1993). We have previously reported that cardiac-specific AngII upregulation induces ventricular hypertrophy and modulates cardiomyocyte electromechanical coupling in male adult mice (Domenighetti*, et al.* 2007; Domenighetti*, et al.* 2005; Gusev*, et al.* 2009). In this transgenic mouse model, intracardiac AngII levels are elevated (Mazzolai*, et al.* 2000) and progression to failure in males involves a transition from compensated, hypertrophic cardiomyopathy to a decompensated, dilated phenotype with altered intracellular Ca^{2+} handling dynamics (Domenighetti*, et al.* 2005). No study to date has investigated these effects in females.

Given that i) conventional heart failure treatments appear less effective in women than in men, ii) female (but not male) cardiomyocytes exhibit suppressed Ca^{2+} current with aging and iii) estrogen-regulation of RAS components is evident, we hypothesised that AngII-induced contractile impairment would be exacerbated in aged female cardiomyocytes linked with Ca^{2+} handling disturbances. To elucidate the sex-specific effects of aging and cardiac AngII elevation on myocardial function, isolated cardiomyocytes from young adult and aged adult male and female mice with genetically manipulated angiotensinogen overexpression (AngII-TG) were evaluated for contractile and Ca^{2+} disturbances. We demonstrate that aging in AngII-TG females is associated with selective reduction in cardiomyocyte contractility. In contrast, cardiomyocytes of aged male AngII-TG maintain contractility but exhibit a marked decrease in myofilament Ca^{2+} responsiveness and heightened vulnerability to spontaneous Ca^{2+} release. This study provides the first mechanistic demonstration that aging induces functionally disparate and sex-specific difference in heart failure progression in a setting of elevated cardiac AngII. The biology of aging impacts on the development of cardiopathology differentially in males and females.

Methods

Animals and dietary treatments

The transgenic mouse line with cardiac overexpression of the angiotensinogen gene (Tg1306/1R) was created by Pedrazzini and colleagues (Lausanne, Switzerland) by the insertion of 30 copies of the rat angiotensinogen transgene into the mouse genome (Mazzolai*, et al.* 1998). The genetic background of these mice is estimated to be ~99% C57Bl/6. All mice were housed in temperature-controlled conditions in a 12hr light/dark cycle, and were cared for in accordance with the 'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and procedures were approved by the Animal Ethics Committee of the University of Melbourne (Approval #0703180 & #1112184). Male and female wildtype and transgenic mice were used at young adult (~13wks old) and aged adult (~87wks old) age ranges.

Cardiomyocyte isolation

Hearts were excised from heparinised (100 I.U, i.p.) and anaesthetised mice (sodium pentobarbitone, 140mg/kg i.p.) and the aorta was cannulated. The heart was retrogradely perfused with Ca^{2+}-free HEPES-buffered Krebs (in mM: 150 NaCl, 5 KCl, 0.33 NaH_2PO_4, 1 $MgCl_2$, 25 HEPES, 20 D-glucose, 3 Na pyruvate, 1 Na lactate, pH 7.4) at 2ml/min at 37°C for 10 minutes. Addition of Type II Collagenase (0.66mg/ml, 295U/mg, Worthington Biochemical Corporation, NJ, USA), $CaCl_2$ (50µM) and Trypsin (33µg/ml; Sigma-Aldrich, MO, USA) to the perfusate enabled heart digestion. The heart was then removed from the cannula, atria dissected away, and the ventricles gently teased apart. Cells were dispersed in a high potassium HEPES-buffered Krebs solution (in mM: 30 KCl, 90 KOH, 30 KH_2PO_4, 3 $MgSO_4$, 50 Glutamate, 20 Taurine, 0.5 EGTA, 10 D-glucose, 10 HEPES, pH 7.4) and resuspended in Ca^{2+}-free HEPES-buffered Krebs with trypsin inhibitor (25µg/ml; Sigma-Aldrich, MO, USA).

Cardiomyocyte Ca^{2+} handling and twitch analysis

Cardiomyocytes were loaded with the Ca^{2+} fluorescent dye, Fura2-AM (1µM, 20min incubation at 25°C; Invitrogen, CA, USA). The Fura2 loading conditions provided an optimal signal-to-noise ratio without compromising myocyte inotropic status and responsiveness. Myocytes were superfused with 2mM Ca^{2+} HEPES-buffer (in mM: 146.2 NaCl, 4.69 KCl, 11 D-glucose,

0.35 $NaH_2PO_4H_2O$, 1.05 $MgSO_47H_2O$, 10 HEPES) at 2.6ml/min and field-stimulated at 4Hz to establish steady state contractile performance (>5min, 37˚C) followed by a low frequency challenge (0.5Hz) for assessment of spontaneous activity. Myocyte Ca^{2+} signals were measured by microfluorimetry (360:380nm fluorescence ratio, 1000Hz sampling; IonOptix, MA, USA) as previously described (Mellor*, et al.* 2012). Ca^{2+} transient timecourse was assessed by measurement of the time constant of decay (Tau; exponential curve set from 50% decay). All fluorescent signals were corrected for background. Cardiomyocyte twitch properties were assessed by video-based edge-detection (IonOptix, MA, USA) and analysed for peak shortening normalised to diastolic cell length (%S), maximum rate of shortening normalised to diastolic cell length (%MRS) and maximum rate of lengthening normalised to diastolic cell length (%MRL). All indices were analysed off-line using IonWizard (IonOptix, MA, USA) and were determined after averaging 10 steady-state transients for each myocyte. Representative Ca^{2+}-shortening phase loop plots were constructed as described (Bers 2001; Spurgeon*, et al.* 1992). The Fura2 signal ($F_{380:360}$) at 50% myocyte relaxation (expressed as % change from diastolic Ca^{2+}) was evaluated as an indicator of myofilament Ca^{2+} responsiveness.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from AngII-Tg and WT male and female mouse cardiac ventricular tissues using the TRIzol® reagent in conjunction with the PureLinkTM Micro-to-Midi Total RNA Purification kit (Invitrogen, CA, USA) as per the manufacturer's instructions and was reverse transcribed using SuperScript™ III First-Strand Synthesis System (Invitrogen, CA, USA). Real-time RT-PCR was used to determine the relative gene expression levels of AngII receptor type 1a ($AT_{1a}R$). Real time RT-PCR primer sequences for AT1aR:
Fwd 5'-TGCCATGCCCATAACCATCTG-3' and Rev 5'-CGTGCTCATTTTCGTAGACAGG-3'.
The comparative ΔΔCt method was utilized to analyze the genes of interest as described (26, 31).

Statistical Analyses

Figures 1, 2 and 6 present raw data from all groups of mice. Figures 3-5 present data as a percentage of the young WT group for each sex. Data are presented as mean ± standard error of the mean (S.E.M). Statistical analyses were performed using SPSS V20 (SPSS Inc., IL, USA). Data were analysed by multi-way ANOVA with post-hoc Fisher's LSD tests where appropriate. Data were log-transformed where appropriate to satisfy the assumption of equal variances. A p-value<0.05 was considered statistically significant.

Results

Animal and cardiomyocyte characteristics

Wildtype and AngII-TG male and female mice were aged-matched for 'young adult' (13 ± 0.1weeks) and 'aged' adult (87 ± 0.6 weeks) groups. The 'young' adult age was chosen on the basis of previously established AngII-induced cardiac hypertrophy at this age (Mazzolai, *et al.* 2000). The 'aged' cohort was chosen to be within the last quartile of C57Bl/6 lifespan for females (average lifespan: females 113wks, males 125wks (Kunstyr and Leuenberger 1975)) and based on our previous report that significant AngII-induced mortality is evident at this age (Domenighetti, *et al.* 2005).

In male WT there was no aging-associated increase in cardiomyocyte length dimension. In young males, cardiomyocytes of AngII-TG mice were elongated ~11% relative to WT mice and cellular hypertrophy was accentuated with age (aged male AngII-TG myocytes were approximately 22% longer than aged male WT). In young female AngII-TG mice, cardiomyocytes were ~14% longer relative to young WT. Interestingly with aging in females, neither WT or AngII-TG myocytes elongated - indeed, myocytes of aged females were marginally (not significantly) shorter than myocytes from young adults (~8% in WT and ~3% in AngII-TG). Thus cardiomyocytes of aged AngII-TG females were observed to be ~20% longer than those of aged female WT mice (Figure 1; diastolic cell length, 2-way ANOVA $p<0.05$). Cardiomyocyte hypertrophy was therefore confirmed for both male and female AngII-TG mice. The degree of cardiomyocyte hypertrophy induced by AngII overexpression increased with age in males due to AngII-TG myocyte enlargement whereas the apparent increase in degree of hypertrophy observed with age in females (20% vs. 14% increase, aged vs. young respectively) partially reflected by an age-associated decrement in myocyte length in WT mice.

Sex-specific effects of aging & cardiac AngII on cardiomyocyte contractility

Representative cardiomyocyte twitch profiles from each group are depicted in Figure 2. Male WT myocytes exhibited a diminished twitch contraction with age (Figure 2A). In the male, no difference was observed in twitch contraction profiles between young and aged AngII-TG cardiomyocytes (Figure 2B), primarily reflecting a reduced contractile state in the young AngII-TG compared with the young WT. Female WT mice exhibited a relatively modest reduction in twitch contraction amplitude with age (Figure 2C). The twitch profile in young adult female myocytes was not

modified by AngII overexpression, but in contrast to males, myocytes of aged AngII-TG females exhibited a marked reduction in the size of the twitch contraction (Figure 2D).

A detailed analysis of aging-related shifts in cardiomyocyte contractile parameters was undertaken to quantify mean group differences in twitch characteristics. The parameters employed to describe the twitch contraction are depicted in Supplementary Material Figure S1-A. The contractile parameters for AngII-TG mouse cardiomyocytes are presented normalised to young adult WT values for each sex (Figure 3). Mean cardiomyocyte % shortening (maximal change in length normalised to resting cell length) was significantly diminished by transgenic cardiac elevation of AngII in young male mice (~72% of young WT levels, $p<0.05$). A similar level of deficit was observed in aged male AngII-TG mice ($p=0.054$ vs. young WT). In myocytes of young females, % shortening was not affected by cardiac AngII elevation but aged female AngII-TG mice exhibited a significant and marked reduction in extent of myocyte shortening (Figure 3A).

Similar patterns emerged for cardiomyocyte twitch kinetics. The maximum rate of myocyte shortening (expressed normalised to resting cell length, %MRS) was reduced in young male AngII-TG myocytes, ~74% of WT values, but this effect did not reach statistical significance ($p=0.086$). Similarly, a trend for reduced %MRS in aged male AngII-TG mice was observed, ~63% of WT values ($p=0.054$). Thus %MRS in aged male AngII-TG myocytes was not different to young male AngII-TG. In AngII-TG female myocytes, %MRS was slightly faster than WT myocytes in the younger mice (difference not significant). In contrast, the aged AngII-TG female myocytes exhibited markedly slower %MRS (~58% of young female WT values, $p<0.05$; Figure 3B). Cardiomyocyte maximum rates of lengthening (expressed normalised to resting cell length, %MRL) was significantly slower in young and aged male AngII-TG mice (~61% and ~51% of young WT values respectively, $p<0.05$; Figure 3C). Young female AngII-TG myocytes however, exhibited preserved relaxation kinetics but an age-related deficit in the rate of lengthening was observed (~44% of WT values, $p<0.05$). These data indicate that the age-dependent contractile response of females to AngII overexpression is distinctly different to males. No early deficit is observed, and a later substantial functional detriment is apparent. In contrast, male myocytes at both ages show an intermediate contractile dysfunction.

Refer to Supplementary Material for full details of contractile parameter data from all groups of mice (Table S1).

Aging selectively delays cytosolic Ca^{2+} removal in female myocytes of high AngII mice

To determine whether the disparate sex effects of aging on cardiomyocyte contractility were associated with underlying Ca^{2+} handling disturbances, Ca^{2+} transients were recorded from myocytes loaded with the intracellular Ca^{2+} fluorphore, Fura 2. Diastolic Ca^{2+} levels were lower in male and female AngII-TG myocytes relative to WT in both age groups, no sex differences were apparent (Supplementary Material Figure S2). The time constant of Ca^{2+} transient decay was similar in male WT and AngII-TG myocytes and was not different with age. In contrast, cardiac AngII overexpression slightly reduced the time constant of Ca^{2+} decay in young female mice (~86% of young WT values) and this parameter was markedly increased in aged female AngII-TG mice (~122% of young WT values, $p<0.05$; Figure 4A). Representative cardiomyocyte Ca^{2+} transients for male AngII-TG young and aged mice are shown in Figure 4B and for female AngII-TG young and aged mice in Figure 4C. The combined effects of aging and elevated cardiac AngII appear to delay cytosolic Ca^{2+} removal selectively in females.

Refer to Supplementary Material for full details of Ca^{2+} transient characteristics from all groups of mice (Table S1) and a schematic depicting the Ca^{2+} transient parameters (Figure S1-B).

Suppressed myofilament responsiveness to Ca^{2+} in aged AngII-TG male cardiomyocytes

Evidence of AngII-induced altered myofilament responsiveness to Ca^{2+} in young adult and aged adult male and female cardiomyocytes was sought. Examination of individual myocyte Ca^{2+}-shortening relationships was undertaken using “phase-loop” analysis (Mellor*, et al.* 2012; Spurgeon*, et al.* 1992). These analyses map myocyte Ca^{2+} levels against cell length (expressed as % change from diastolic Ca^{2+} and cell length respectively) throughout the contraction and relaxation phases of the activation cycle. During the relaxation phase, the descending portion of the “loop” provides a dynamic index of myofilament Ca^{2+} sensitivity (Bers 2001; Spurgeon*, et al.* 1992). Exemplar myocyte Ca^{2+}-shortening phase-loop plots show a right shift in the relaxation phase in the aged male AngII-TG compared with young adult male AngII-TG (Figure 5A), indicative of decreased myofilament responsiveness to Ca^{2+} (Spurgeon*, et al.* 1992). Young and aged female AngII-TG myocytes exhibit similar Ca^{2+}-shortening phase-loop plots (Figure 5B). The phase-loop shift was quantified by assessment of intracellular Ca^{2+} at 50% myocyte relaxation (expressed relative to the diastolic Ca^{2+} level). Aged male AngII-TG mouse cardiomyocytes exhibited a ~70% increase in Ca^{2+} levels at 50% myocyte relaxation relative to young male AngII-TG ($p<0.05$; Figure 5C). Female cardiomyocytes did not exhibit significant changes in Ca^{2+} at 50% myocyte relaxation

with age or AngII overexpression. These data are consistent with an age-related decrease in myofilament Ca^{2+} responsiveness in male cardiomyocytes, but not female cardiomyocytes, in a setting of elevated cardiac AngII.

Aged female cardiomyocytes with elevated cardiac AngII exhibit lower spontaneous activity

Progression to failure and arrhythmogenic predisposition is frequently linked with distrurbed cardiomyocyte diastolic Ca^{2+} handling. Thus, evidence of an aging-related, sex-specific difference in cardiomyocyte diastolic Ca^{2+} management was sought. Manipulation of diastolic interval was used to potentiate intracellular sarcoplasmic reticulum store Ca^{2+} uptake and evaluate Ca^{2+} overload predisposition. Myocyte pacing at a subphysiological frequency (0.5Hz), and at physiological temperature (37C), was employed as a low frequency challenge to evaluate the emergence of spontaneous (i.e. potentially arrhythmogenic) myocyte activity. The proportion of male WT cardiomyocytes that exhibited spontaneous Ca^{2+} release at 0.5Hz stimulation increased with age (Figure 6A). 47% of young male myocytes with cardiac AngII overexpression were spontaneous at low frequency compared with 22% of young male WT myocytes. Young and aged AngII-TG male myocytes exhibited similar levels of spontenous activity. More young female WT myocytes (40%) exhibited spontaneous activity than young male WT myocytes but surprisingly, 90wk old female myocytes with elevated cardiac AngII had the lowest proportion of cells exhibiting spontaneous activity (17%) of all groups (Figure 6A). Representative twitch and Ca^{2+} traces depicting the relative levels of spontaneous activity in young adult and aged adult female AngII-TG myocytes are presented in Figure 6B. The time to first spontaneous Ca^{2+} release was not significantly different between groups (Table S2). These data suggest that the female cardiomyocyte adaptations to age and AngII may act to stabilize the sarcoplasmic reticulum Ca^{2+} store during a low frequency challenge.

Sex-specific AngII-induced cardiomyocyte dysfunction is not linked to altered $AT_{1a}R$ expression

To determine whether sex differences in cardiomyocyte function observed with high AngII at the young adult stage were due to differences in the receptor for AngII, mRNA expression of $AT_{1a}R$ was measured in heart tissue of AngII-Tg and WT male and female young adult mice using real time RT-PCR. Assessment of mRNA expression (vs. protein expression) is the preferred option for AngII receptors due to limitations relating to antibody specificity. $AT_{1a}R$ mRNA expression was similar in all groups suggesting that the sex-specific effects of elevated cardiac AngII on cardiomyocyte function were not linked to altered expression of the $AT_{1a}R$.

Discussion

This is the first investigation to demonstrate distinctly different patterns of 'functional aging' in cardiomyocytes in the presence and absence of genetic influence of heightened cardiac RAS activity. Comparing adult cellular functional phenotype in cardiomyocytes of rodents in the first and last life-span quartiles, these data provide novel evidence that aging is selectively associated with reduced contractility of female (but not male) cardiomyocytes when cardiac AngII is chronically elevated. Specifically, in mice genetically manipulated to overexpress angiotensinogen in the heart, aging was associated with decreased extent of myocyte shortening and slower twitch contraction and relaxation rates in females. These kinetic shifts in myocytes of aged females were observed concomitant with delayed cytosolic Ca^{2+} removal during the relaxation phase of the twitch cycle and preserved myofilament responsiveness to Ca^{2+}. In contrast, in male cardiomyocytes, AngII overexpression caused a similar extent of contractile suppression in young and aged myocytes. The observation that the contractile deficit in male myocytes was not accentuated with aging suggests that aging and AngII act by a convergent mechanism in male but not female myocytes. Myofilament Ca^{2+} responsiveness was reduced with age in male AngII-TG myocytes only, yet contractility was preserved. In males, an expected increase in spontaneous activity was observed with age and cardiac AngII overespression, but surprisingly, aged female AngII-TG myocytes display a remarkable stability of SR Ca^{2+} stores during a low frequency challenge. Female myocytes with elevated AngII appear more susceptible than male myocytes to an age-related contractile deficit whereas male AngII-TG myocytes preserve contractile function with age but exhibit desensitisation of myofilaments to Ca^{2+} and a heightened vulnerability to spontaneous activity. These findings validate the hypothesis advanced.

Sex-specific progression of AngII-induced cellular hypertrophy with aging

Cardiac renin-angiotensin system upregulation has been identified clinically and experimentally in settings of aging-associated cardiopathology. Cardiac hypertrophy is a well established structural phenotype linked with AngII elevation. The cardiac-specific angiotensinogen overexpressing transgenic mouse is a normotensive model of cardiac hypertrophy previously characterised to exhibit *in vivo* cardiac dysfunction and cardiomyocyte electromechanical coupling abnormalities evident by 15-20weeks of age and transition to dilated failure with ~45% reduced mortality at 94weeks (Domenighetti, *et al.* 2005; Gusev, *et al.* 2009). Using this model it is possible to investigate the direct hypertrophic and functional impacts of AngII excess. Cardiomyocyte

hypertrophy was established in myocytes from aged AngII-TG animals, with the differential in myocyte size relatively similar in male and female cell populations (21-23% myocyte elongation, Figure 1). This is consistent with our previous finding that the extent of cardiac hypertrophy is similar in male and female AngII-Tg mice (Domenighetti, *et al.* 2005; Huggins, *et al.* 2009). There is indication (non-significant finding) that in males the pro-growth AngII influence is more marked with aging, whereas for female myocytes the effect of local AngII offsets a reduction in myocyte size that occurs with aging in WT. This may suggest that there are underlying sex differences in aging trophic responses in wildtype mice, and this requires further investigation mechanistically. It has been previously reported that myocyte hypertrophy in this transgenic model (male mice at 8 and 20 weeks of age) is driven by an AngII-induced intrinsic mitogen-activated protein kinase (MAPK) signalling activation, rather than systemic influence (Pellieux, *et al.* 2000). Whether this effect is mediated by autocrine/paracrine actions of AngII at sarcolemmal receptors (subtypes AT_1R, AT_2R) has not been determined. Baker *et al.* demonstrated that AngII has intracrine effects in neonatal cardiomyocytes and *in vivo* mouse hearts (Baker, *et al.* 2004) and receptor-independent intracellular AngII actions on myocyte functional remodelling may play a role in the present study.

Cardiac AngII-induced contractile dysfunction is age-dependent in female myocytes

In the setting of elevated cardiac AngII, we determined that myocyte contractility in female mice was markedly reduced with age. AngII elevation was associated with functional deficit in young and aged male myocytes but age *per se* did not confer additional detriment (Figure 2). These findings demonstrate that there are fundamental differences in the mechanistic progression of cardiac aging and functional deterioration in males and females. A significant reduction in cardiomyocyte extent of twitch shortening and rate of relaxation by cardiac AngII elevation was observed in male mice but young and aged AngII-TG myocytes exhibited similar contractile deficit indicating that aging and AngII may act via a convergent mechanism in male myocytes. These data are consistent with our previous study which demonstrated that significant detriment in cardiomyocyte contractile kinetics is observed in male AngII-TG, evident at the cellular and *ex vivo* heart level in the absence of fibrosis (Domenighetti, *et al.* 2005; Huggins, *et al.* 2003). In the present study, a trend for lower extent of shortening and slower shortening kinetics was observed in young female vs. male WT cardiomyocytes (Supplementary Material Table S1). These data are consistent with findings reported in the literature (Ceylan-Isik, *et al.* 2011; Howlett 2010). In contrast to Ceylan-Isik *et al* (but consistent with Howlett et al), we did not observe a difference in the time to peak myocyte shortening in young female and male myocytes. In wildtype mice,

Howlett *et al* reported that age-related cardiomyocyte functional impairment was less marked in female mice than male mice (Howlett 2010). Our findings extend this work by demonstrating that age-related female myocyte functional detriment emerges in a setting of high cardiac AngII, to a level which is comparable with male. At the young adult stage, when sex-differences in AngII-Tg cardiomyocyte function are most marked, $AT_{1a}R$ expression is not different in male or female AngII-Tg vs. WT hearts suggesting that the sex-specific effects of AngII are not associated with changes in receptor density.

The cardiac renin-angiotensin system is known to mediate many of the remodelling characteristics of heart failure and has also been shown to progressively increase in activity with age (Heymes*, et al.* 1998; Heymes*, et al.* 1994; Unger and Li 2004). Elevated cardiac AngII suppresses the fatty acid oxidation pathway and reduces insulin-dependent glucose transporter 4 (GLUT4) expression, albeit with a concomitant increase in GLUT1 (Pellieux*, et al.* 2006). Thus the provision and metabolism of both glycolytic and non-glycolytic substrates for ATP supply may be limiting in relation to maintenance of contractile function when cardiac AngII is chronically elevated. We have previously reported that AngII-TG male mice exhibit altered cardiomyocyte Ca^{2+} handling at 15-20wks of age. Reduced expression of SERCA2a, the ATP-dependent transporter responsible for loading/reloading activator Ca^{2+} from the cytosol into the sarcoplasmic reticulum stores for release during each twitch cycle is observed, associated with lower sarcoplasmic reticulum Ca^{2+} load (Domenighetti*, et al.* 2005; Gusev*, et al.* 2009). Similarly, AT1R overexpression is linked with a reduction in cardiac SERCA2 expression (Rivard*, et al.* 2011). There is evidence that SERCA2 function may be specifically modulated by glycolytically-derived ATP (Kockskamper*, et al.* 2005). In the present study, we observed delayed Ca^{2+} removal from the cytosol during twitch relaxation in aged AngII-TG female myocytes. This finding is consistent with AngII-induced SERCA2 downregulation and may explain the slower rate of twitch relaxation observed in this group (Figure 2C). Recently, sex differences in cardiomyocyte Ca^{2+} handling have been reviewed in detail (Bell*, et al.* 2013; Parks and Howlett 2013). In wildtype mice, SERCA2 expression has been reported to be not different or lower in female hearts relative to male (Ceylan-Isik*, et al.* 2011; Parks and Howlett 2013). The effect of ovariectomy on SERCA2 expression decreases SERCA2 expression only with long-term hormone withdrawal (10 weeks duration (Bupha-Intr*, et al.* 2009; Bupha-Intr and Wattanapermpool 2006)). Treatment of H9c2 cells with 17β-estradiol increases SERCA2 expression (Liu*, et al.* 2007). Findings from the present study indicate that with aging, female cardiomyocytes become more vulnerable to sarcoplasmic reticulum Ca^{2+} uptake impairment, a condition which has been generally

linked to diastolic dysfunction (Asp, *et al.* 2012). Age-related estrogen-decline in females may underlie a sex-specific susceptibility to Ca^{2+} mishandling with high AngII and warrants further investigation.

Cardiac AngII elevation selectively decreases myofilament Ca^{2+} responsiveness in aged males

Aged male cardiomyocytes with elevated cardiac AngII exhibited a marked decrease in cardiomyocyte myofilament responsiveness to Ca^{2+}, demonstrated by a right shift in the Ca^{2+}-shortening phase loop plot (Figure 5A). This effect was not observed in females (Figure 5B). No previous study has investigated the effect of age and chronic cardiac AngII elevation on cardiomyocyte Ca^{2+} sensitivity. Interestingly, these findings contrast with the known myofilament 'sensitisation' to Ca^{2+} that occurs with acute AngII exposure by *ex vivo* perfusion (Ikenouchi, *et al.* 1994; Mattiazzi 1997). An early study using skinned cardiomyocyte preparations investigated myofilament Ca^{2+} sensitivity in aged male rats and demonstrated that, although myofibrillar ATPase activity was reduced relative to young adult rats, no change in Ca^{2+} sensitivity was observed (Bhatnagar, *et al.* 1984). The findings from the present study suggest that elevated cardiac AngII reduces cardiomyocyte Ca^{2+} sensitivity with age in male mice only. The sex-specificity of this action is very marked. While myocytes of both young and aged females preserved a Ca^{2+}-responsiveness equivalent to young WT, in the male aged AngII-TG there was a 70% upward shift in the Ca-shortening relationship (Figure 5C). These findings suggest that aging and cardiac AngII act synergistically to suppress Ca^{2+} sensitivity in male cardiomyocytes only. The mechanism by which aging and chronic AngII elevation modulate myofilament Ca^{2+} responsiveness is not clear but may involve a reduction in pH_i, shift in the α:β-myosin heavy chain ratio or an increase in troponin-I phosphorylation. We have previously reported that an age-dependent reduction in Na^+-H^+ exchanger expression is evident in AngII-TG male mouse hearts (Domenighetti, *et al.* 2001) thus impaired extrusion of intracellular H^+ ions may contribute to a decrease in pH_i and consequent decrease in myofilament Ca^{2+} responsiveness. Other investigators have identified a role for AngII-induced phosphorylation of troponinI in reducing Ca^{2+} sensitivity in hyperglycaemic cardiomyocytes *in vitro* (Malhotra, *et al.* 2001). Whether this AngII action can be extrapolated to other disease settings (i.e. aging-associated chronic AngII elevation) has not yet been established.

Spontaneous activity is attenuated with age in female AngII-TG myocytes

In this study it is demonstrated that with aging, male (but not female) WT cardiomyocytes exhibit increased spontaneous Ca^{2+} release. Young male AngII-TG myocytes also exhibit a similar increase in spontaneity relative to WT - but in the aged AngII-TG there is no compounding of the effects of AngII and age to exacerbate the level of spontaneous activity (Figure 6). It has been previously shown that cardiac angiotensinogen overexpression induces a prolonged QT interval coincident with reduced expression of IK1-related KCNJ2 and KCNJ12 potassium channels in male mice at 50-60 weeks of age. Isolated cardiomyocytes from these male AngII-Tg mice also exhibited prolonged repolarization phase of the action potential. This earlier study reported that male transgenic mice are more susceptible to arrhythmias, as measured by *in vivo* ECG analysis (Domenighetti*, et al.* 2007). Electrical disturbances may partially underlie our finding of heightened spontaneous activity in isolated male AngII-Tg cardiomyocytes. Additionally, the observed increased time to peak Ca^{2+} transient in male AngII-Tg myocytes may reflect prolonged Ca^{2+} release from the SR by ryanodine receptors, previously shown to contribute to arrhythmogenesis (Gomez and Richard 2004). Diastolic Ca^{2+} levels were lower in AngII-TG mice relative to WT in both age groups thus spontaneous activity is not related to elevated cytosolic Ca^{2+} during diastole.

Interestingly, female myocytes exhibit a markedly different response. Aged female myocytes with elevated cardiac AngII exhibit lower spontaneous activity in association with a marked contractile deficit. In females, irregular Ca^{2+} release from the SR may be prevented by the observed AngII-induced electromechanical coupling alterations (in particular, the prolonged Ca^{2+} transient observed in aged female AngII-TG mice), likely indicative of suppressed sarcoplasmic reticulum Ca^{2+} loading. It is notable that the marked reduction in spontaneous activity is observed in the presence of prolonged Ca^{2+} release (increased time to peak Ca^{2+} transient) suggesting that female aged AngII myocytes may exhibit compensatory mechanisms to prevent spontaneity in this pro-arrhythmogenic setting. Our previous study reported that female AngII-Tg mice (34 wk) exhibit significantly less % ectopic beats relative to female WT mice during the first 10 min reperfusion post-ischemia. Detailed analysis of arrhythmic events revealed that female AngII-Tg mice exhibited lower incidence of ventricular premature beats and ventricular tachycardia and shorter duration of bigeminy relative to female WT mice. Ventricular fibrillation was detected in female WT hearts but not AngII-Tg hearts (Huggins*, et al.* 2009). The lower level of spontaneous activity observed in aged female AngII-Tg myocytes in the present study is consistent with the lower incidence of arrhythmias identified *in vivo* in Huggins *et al* 2009. Interestingly, these findings are consistent with

clinical observations of arrhythmia-related mortality. The Framingham Heart Study reported that women with heart failure have lower incidence (approximately one quarter) of sudden cardiac death than men (Kannel*, et al.* 1998). Thus female-specific cardiac functional adaptations in aged failing hearts may limit occurrence of arrhythmogenic events - yet confer failure liability.

In conclusion, this is the first study to demonstrate that cardiomyocyte functional state deteriorates with age in a sex- and AngII-dependent manner. Female myocytes appear more susceptible to an age-related contractile deficit in a setting of high cardiac AngII, but exhibit a low level of spontaneous activity. In contrast, aged male myocytes exposed to elevated cardiac AngII maintain contractile function relative to young adult myocytes but a profound desensitisation of myofilaments to Ca^{2+} is apparent, associated with a heightened vulnerability to arrhythmic activity. The findings from this study provide important mechanistic insight into the progression of aging- and AngII-related cardiac pathology in males and females and support the contention that sex-specific therapies are required for the treatment of age-progressive heart failure. This may be particularly relevant to the clinical targeted use of AngII-directed pharmacological intervention.

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Supplementary material

Additional supplementary material may be found in the online version of this article:

Table S1. Cardiomyocyte functional characteristics of young and aged adult male and female WT and AngII-TG mice.

Table S2. Time to first spontaneous Ca^{2+} release during 0.5Hz stimulation in cardiomyocytes from young and aged adult male and female WT and AngII-TG mice.

Figure S1. Schematic of parameter definitions.

Figure S1. Diastolic Ca^{2+} levels.

Figure Legends

Figure 1. Diastolic cardiomyocyte length. Young adult and aged adult male and female cardiomyocytes from wildtype (open circles) and AngII-TG (closed circles) mice. Individual data points (left) and mean ± SEM (right) for each group. n=11-15cells/group. *p(genotype)<0.05, 2-way ANOVA.

Figure 2. Cardiomyocyte twitch shortening profiles are modulated by age in AngII-Tg male and female mice. Representative twitch profiles of young and aged adult male WT myocytes (A), young and aged adult male AngII-TG myocytes (B), young and aged adult female WT (C), young and aged adult female AngII-TG myocytes (D).

Figure 3. AngII-TG contractile performance - age and sex specific shifts compared to young adult WT. A Extent of twitch shortening normalised to diastolic length expressed as a percentage of young adult WT for each sex. **B.** maximum rate of shortening (MRS) normalized to cell length (L_0), expressed as a percentage of young adult WT for each sex. **C.** maximum rate of lengthening (MRL) normalized to cell length (L_0), expressed as a percentage of young adult WT for each sex. n=11-14cells/group. Data presented mean ± S.E.M. *p(age)<0.05, p(interaction)<0.05 2-way ANOVA.

Figure 4. Age prolongs cardiomyocyte Ca^{2+} transient decay in AngII-TG female mice. Cardiomyocytes were loaded with 1μM Fura2 Ca^{2+} dye to evaluate Ca^{2+} cycling. **A.** Ca^{2+} transient time constant of decay (ms), expressed as a percentage of young adult WT for each sex.
B. Representative cardiomyocyte Ca^{2+} transient profiles for young and aged adult male AngII-TG mice. **C.** Representative cardiomyocyte Ca^{2+} transient profiles for young and aged adult female AngII-TG mice. Traces have been normalised to match amplitude to enable direct timecourse comparisons. n=9-11cells/group. Data presented mean ± S.E.M. *p(genotype)<0.05, p(interaction)<0.05 2-way ANOVA.

Figure 5. Myofilament Ca^{2+} responsiveness decreases with aging in AngII-TG male mice only. **A.** Representative Ca^{2+}-shortening phase loops from young and aged male AngII-TG mouse cardiomyocytes. Dashed arrow shows the direction of twitch cycle, solid arrow highlights the right shift of the phase loop. **B.** Ca^{2+}-shortening phase loops from young and aged female AngII-TG mouse cardiomyocytes. Dashed arrow shows the direction of twitch cycle. **C.** Intracellular Ca^{2+}

change from diastolic Ca^{2+} at 50% myocyte relaxation, expressed as percentage change from young adult wildtype for each sex. n=7-11cells/group. Data presented mean ± S.E.M. *p(age)<0.05, $^{\#}$p(sex)<0.05 2-way ANOVA.

Figure 6. Incidence of spontaneous activity emergent at 0.5Hz stimulation. A. The percentage of cells that exhibited spontaneous activity (i.e. non-stimulated) when subjected to a low frequency stimulation protocol (0.5Hz stimulation). **B.** Representative traces for young adult female AngII-TG and aged adult female AngII-TG cardiomyocytes stimulated at 0.5Hz. Arrows depict stimulus.

Figure 7. AngII type 1a receptor mRNA expression in cardiac ventricular tissue. $AT_{1a}R$ mRNA expression in AngII-Tg and WT young adult male and female mouse cardiac ventricular tissue, n=6-8. Data presented mean ± S.E.M.

Supplementary Material File:

Figure S1. Schematic of parameter definitions. A. Cardiomyocyte twitch example with key parameters indicated. **B.** Cardiomyocyte Ca^{2+} transient example with key parameters indicated.

Figure S2. Diastolic Ca^{2+}levels. A. Young male and female WT and AngII-TG cardiomyocyte diastolic Ca^{2+}, expressed relative to young male WT. **B.** Aged male and female WT and AngII-TG cardiomyocyte diastolic Ca^{2+}, expressed relative to aged male WT. Data presented mean ± S.E.M. *p(age)<0.05, 2-way ANOVA.

Abstract

Clinically, heart failure is an age-dependent pathological phenomenon and displays sex-specific characteristics. The renin-angiotensin system mediates cardiac pathology in heart failure. This study investigated the sexually dimorphic functional effects of aging combined with AngII on cardiac muscle cell function, twitch and Ca^{2+} handling characteristics of isolated cardiomyocytes from young (~13weeks) and aged (~87weeks) adult wildtype (WT) and AngII-transgenic mice. We hypothesised that AngII-induced contractile impairment would be exacerbated in aged female cardiomyocytes and linked to Ca^{2+} handling disturbances.AngII-induced cardiomyocyte hypertrophy was evident in young adult mice of both sexes and accentuated by age (aged adult:~21-23% increase in cell length relative to WT). In female AngII-TG mice, aging was associated with suppressed cardiomyocyte contractility (%shortening, maximum rate of shortening, maximum rate of relaxation). This was associated with delayed cytosolic Ca^{2+} removal during twitch relaxation (Tau:~20% increase relative to young adult female WT) and myofilament responsiveness to Ca^{2+} was maintained. In contrast, aged AngII-TG male cardiomyocytes exhibited peak shortening equivalent to young TG, yet myofilament Ca^{2+} responsiveness was profoundly reduced with aging. Increased pro-arrhythmogenic spontaneous activity was evident with age and cardiac AngII overexpression in male mice (42-55% of myocytes), but relatively suppressed in female aged transgenic mice. Female myocytes with elevated AngII appear more susceptible to an age-related contractile deficit whereas male AngII-TG myocytes preserve contractile function with age but exhibit desensitisation of myofilaments to Ca^{2+} and a heightened vulnerability to arrhythmic activity. These findings support the contention that sex-specific therapies are required for the treatment of age-progressive heart failure.

Figure 1.

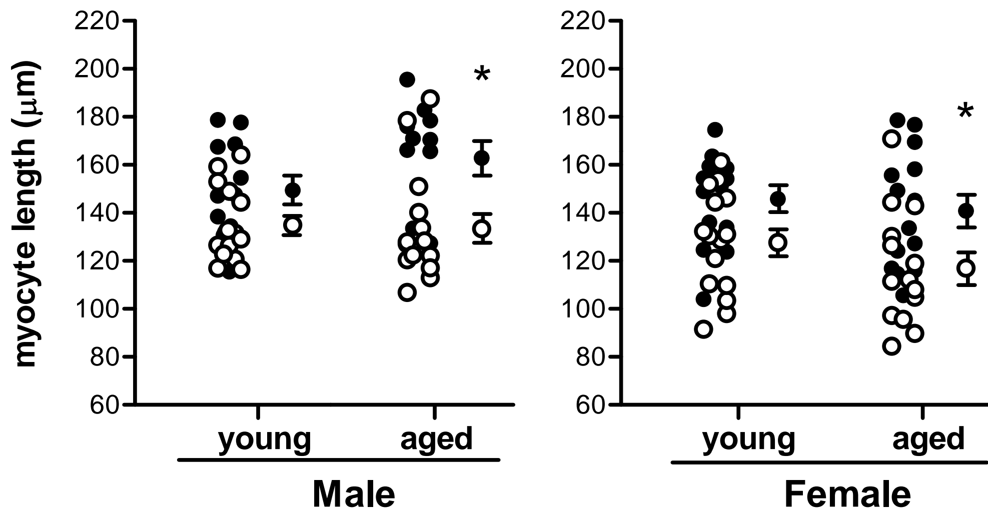

Figure 2.

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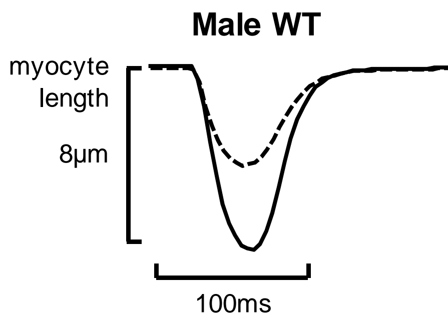

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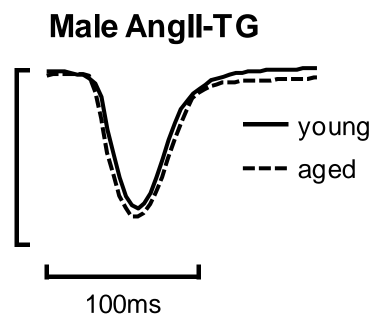

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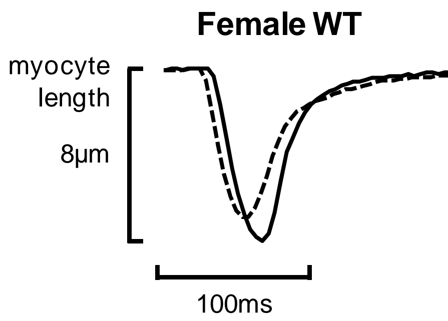

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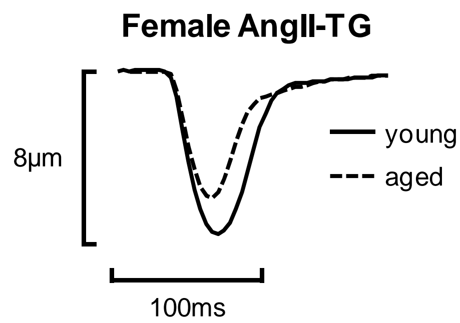
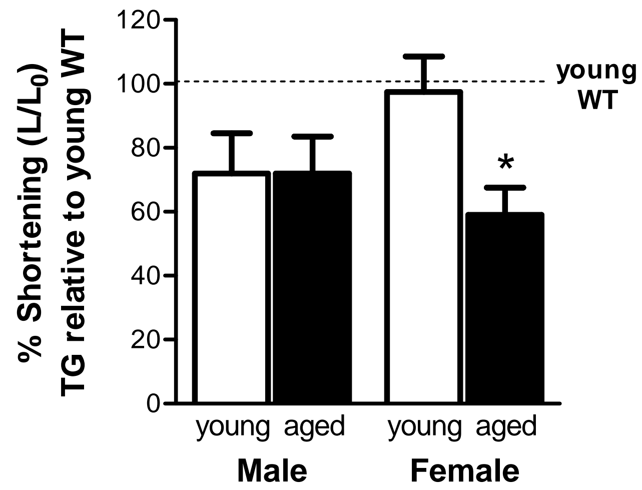


Figure 3.

A.



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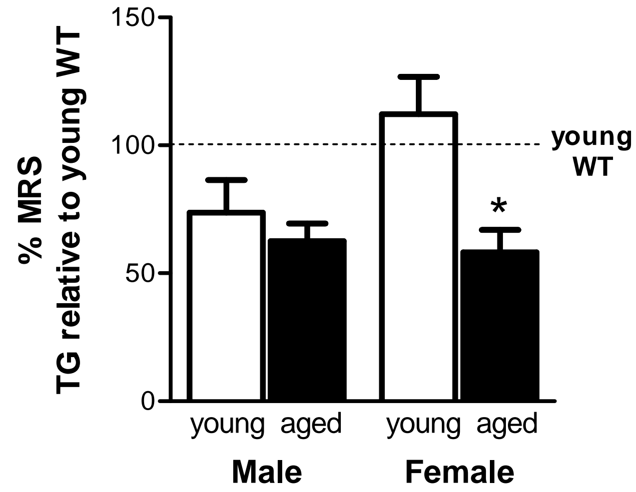

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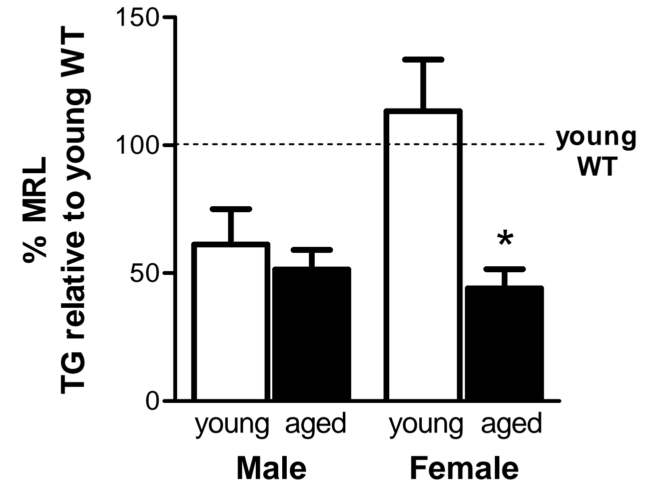


Figure 4.

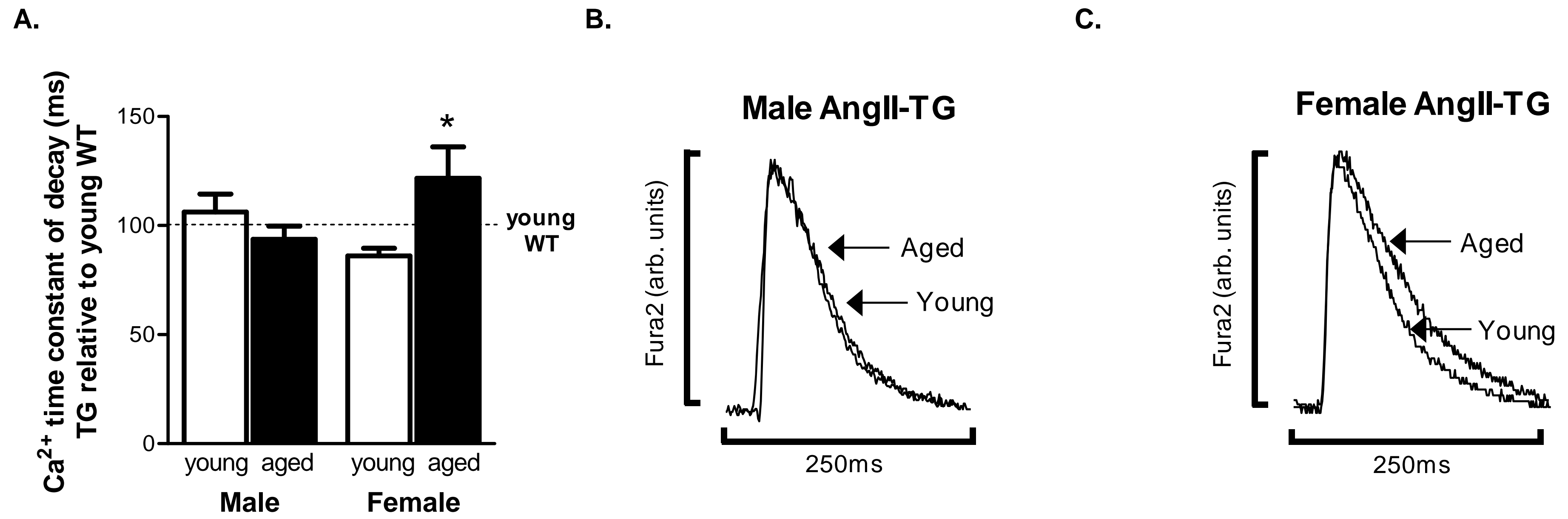


Figure 5.

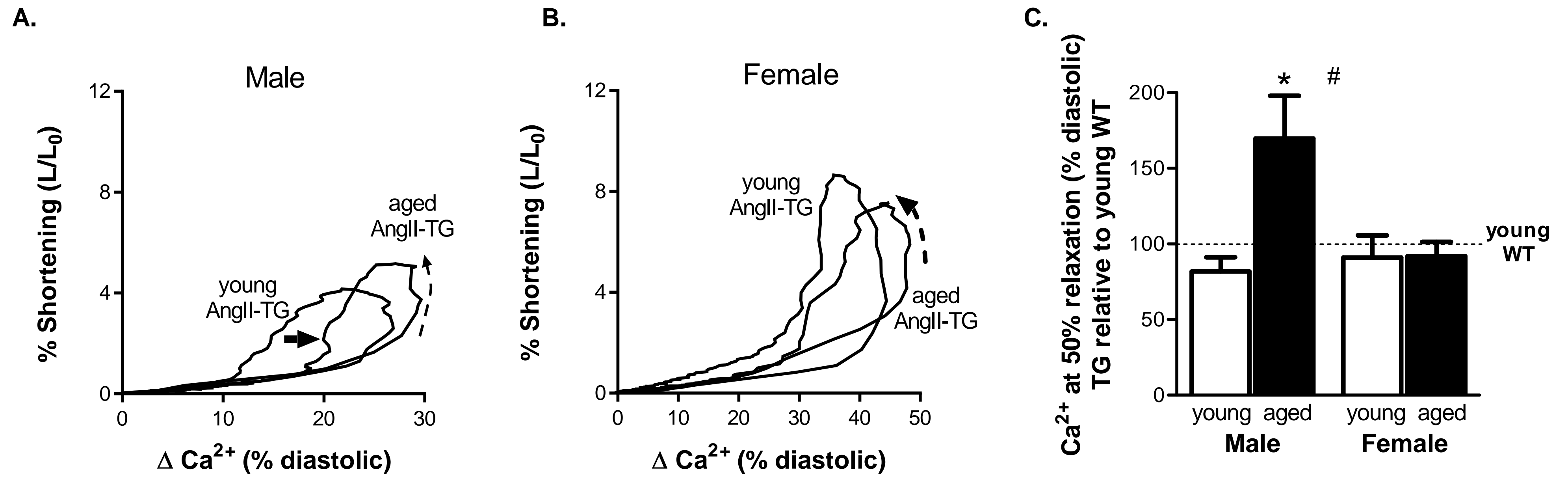
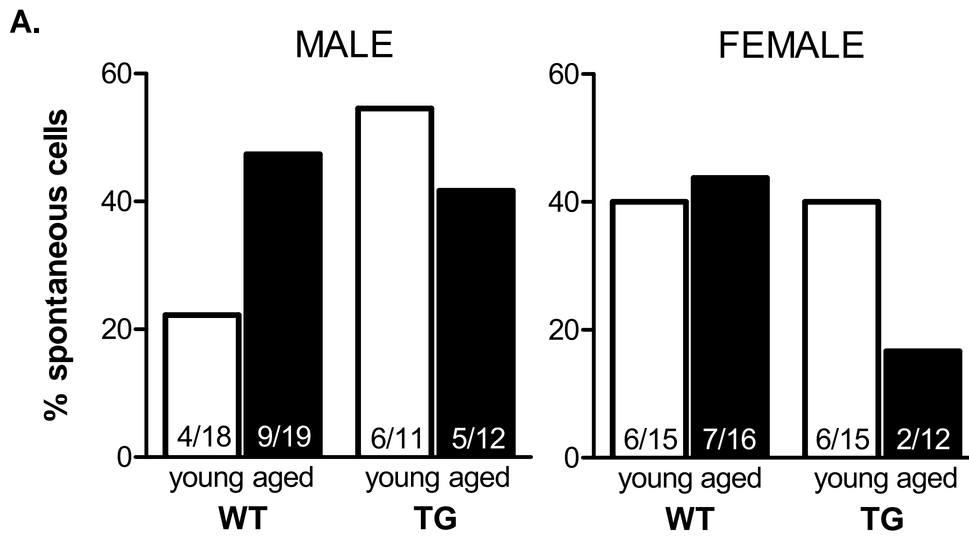


Figure 6.

A.



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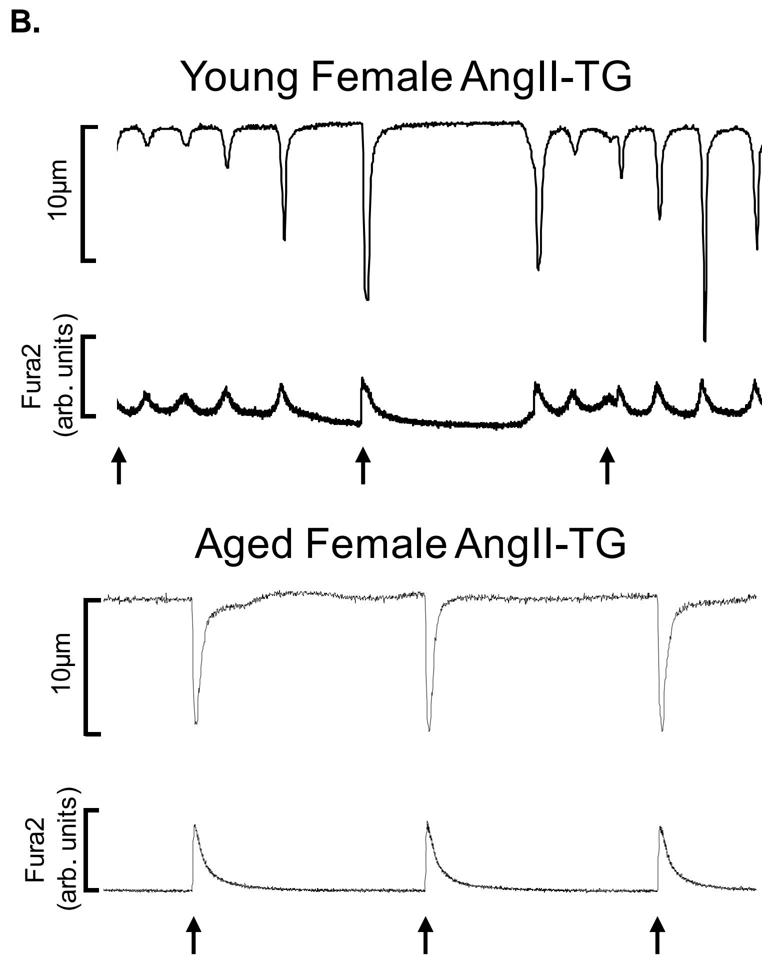

Figure 7.