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Increased CaMKII activation and contrast changes of cardiac β1-and β3-Adrenergic signaling pathways in a humanized angiotensinogen model of hypertension

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ABSTRACT

Aims: Upregulation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) contributes to the pathogenesis of cardiovascular disease, including hypertension. Transgenic rats expressing the human angiotensinogen gene [TGR (hAGT)L1623] are a new novel humanized model of hypertension that associates with declines in cardiac contractile function and β-adrenergic receptor (AR) reserve. The molecular mechanisms are unclear. We tested the hypothesis that in TGR (hAGT)L1623 rats, left ventricular (LV) myocyte CaMKIIδ and β_3-AR are upregulated, but β_1-AR is down-regulated, which are important causes of cardiac dysfunction and β-AR desensitization.

Main methods: We compared LV myocyte CaMKIIδ, CaMKIIδ phosphorylation (at Thr287) (pCaMKIIδ), and β_1-and β_3-AR expressions and determined myocyte functional and $[Ca^{2+}]_I$ transient ($[Ca^{2+}]_{iT}$) responses to β-AR stimulation with and without pretreatment of myocytes using an inhibitor of CaMKII, KN-93 (10^{-6} M, 30 min) in male Sprague Dawley (SD; N = 10) control and TGR (hAGT)L1623 (N = 10) adult rats.

Key findings: Hypertension in TGR (hAGT)L1623 rats was accompanied by significantly increased LV myocyte β_3-AR protein levels and reduced β_1-AR protein levels. CaMKIIδ phosphorylation (at Thr287), pCaMKIIδ was significantly increased by 35%. These changes were followed by significantly reduced basal cell contraction (dL/dt_{max}), relaxation (dR/dt_{max}), and $[Ca^{2+}]_{iT}$. Isoproterenol (10^{-8} M) produced significantly smaller increases in dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$. Moreover, only in TGR (hAGT)L1623 rats, pretreatment of LV myocytes with KN-93 (10^{-6} M, 30 min) fully restored normal basal and isoproterenol-stimulated myocyte contraction, relaxation, and $[Ca^{2+}]_{iT}$.

Significance: LV myocyte CaMKIIδ overactivation with associated contrast changes in β_3-AR and β_1-AR may be the key molecular mechanism for the abnormal contractile phenotype and β-AR desensitization in this humanized model of hypertension.

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Abbreviations

AGT	angiotensinogen
hAGT	human angiotensinogen
TGR(hAGT)L1623	transgenic rats expressing the human angiotensinogen gene in their genome
Ang II	angiotensin II
RAS	renin-angiotensin system
β-AR	β-adrenergic receptor
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
SA	myocyte percent shortening
dL/dtmax	maximum rate of myocyte shortening
dR/dtmax	maximum rate of myocyte re-lengthening
$[Ca^{2+}]_{iT}$	calcium transient
HF	Heart Failure

1. Introduction

Hypertension, a chronic, age-related disorder, remains a leading risk factor for cardiovascular morbidity and mortality. By imposing an increased workload on the heart, the increased afterload is recognized as a risk factor for heart failure (HF) [1,2]. Despite significant advances in the clinical recognition of hypertension-associated mechanisms contributing to HF progression, achieving newer therapeutic milestones remains critically dependent on the availability of animal models mimicking the pathogenic features of the syndrome in humans [3].

Small animal models expressing renin-angiotensin system (RAS) genes represent a critical niche for studying HF mechanisms as this complex blood-borne and tissue hormonal system is an obligatory contributor to cardiac functional impairment and adverse structural remodeling in HF [4]. Transgenic rats expressing the human AGT gene [TGR (hAGT) L1623] represent a novel humanized model of hypertension [5,6]. These transgenic rats were created by Ganten and collaborators [7] as the generation of rats with genomic insertion of both the hAGT and human renin genes (double transgenic rats) [8,9]. The creation of a rat expressing the human AGT gene in its genome allows for exploring non-renin mechanisms of excess Ang II activity as rat renin does not cleave the human AGT protein. We [5,6] and others [7–10] have performed serial functional studies in rats expressing the human AGT gene and reported collective characterization of systemic hemodynamics, transthoracic echocardiography, and rat and human expressions of renin-angiotensin system components in the blood and cardiac tissue [5,6]. We have shown that transgenic rats [TGR (hAGT)L1623] exhibit sustained hypertension, and cardiac hypertrophy, associated with significant increases in cardiac Ang-(1–12) immunofluorescence and a 4-fold increase in cardiac Ang II content [5]. Since endogenous rat renin does not cleave human angiotensinogen (hAGT), this humanized model of hypertension provides a unique opportunity to characterize non-renin-dependent mechanisms for systemic and cardiac Ang II generation through endogenous Ang-(1–12) processing [5,6]. Recently we further found that this model is associated with declines in left ventricular (LV) contractile function and β-adrenergic receptor (AR) reserve [11,12]. Hypertension, most pronounced in TGR (hAGT)L1623 males, includes altered cardiac contractility, and down-regulation of cardiac Ca^{2+} mobilization [5,6, 13–15].

Disrupted cardiomyocyte Ca^{2+} homeostasis contributes to hypertrophic and HF phenotypes. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) represents a nodal point in regulating intracellular Ca^{2+} handling, ion channels, and gene transcription [16–19]. One key mediator of cardiac contractility is the myocardial isoform of CaMKIIδ, which regulates proteins associated with cardiac Ca^{2+} flux [20]. Ang II increases CaMKII activation, which plays a crucial role in Ang II-related pathological processes associated with hypertension, hypertrophy, and cardiac dysfunction [21]. Therefore, CaMKIIδ overactivation may play a vital role in cardiac dysfunction in this model. Although CaMKII is credited to play a major role in the development of heart failure (HF) induced by stresses including hypertension (e.g., pressure-overload by transverse aortic constriction and angiotensin II) [19,22–24], the physiological role of CaMKII is not well defined. Both detrimental and beneficial cardiac effects of CaMKII inhibition have been reported [19,20,24–30]. Furthermore, the functional significance of cardiac CaMKII expression and activation in hypertension remains to be established given the inconsistent conclusions of published studies. In addressing these knowledge gaps, we have undertaken the first direct analysis of CaMKII inhibition on cardiac function and cardiac reserve in transgenic hypertensive TGR (hAGT)L1623 rats.

It is well established that β_1-, β_2-, and β_3-ARs coexist in animals and human hearts. β_3-ARs differ from classic β_1-and β_2-ARs in molecular structure and pharmacological profile, their activation at higher catecholamine concentrations, and β_3-ARs relatively resistant to chronic, agonist-induced desensitization [31–34]. We and others have shown previously that unlike β_1-and β_2-ARs, the function of β_3-AR is to inhibit ventricular contraction through its link to the inhibitory G (G_i) proteins and endothelial nitric oxide (NO) dependent and un-dependent mechanism [35–37]. The β_3-AR is increasingly recognized as a key regulator of cardiac function in both normal and diseased states. Growing evidence indicates that cardiac β_3-AR overactivity promotes progressive cardiac deleterious remodeling and dysfunction in HF and cardiomyopathies [31,38,39]. Recent evidence suggests that a pivotal restructuring of the β-adrenergic system is a critical determinant of the dysfunctional β-AR regulation in the diseased heart. Hypertension is associated with increased cardiac expression of G_i proteins [40–42]. Although β_3-AR stimulation could play an important role in the impaired cardiac β-AR reserve of hypertension, its participation in the adverse cardiac remodeling described in this Ang II-dependent model of

humanized hypertension remains undefined. Furthermore, no data exists as to whether CaMKII represents a possible downstream mediator of detrimental β-AR signaling contributing to the abnormal contractile phenotype of cardiac hypertrophy [43,44].

With this in mind, we have tested the hypothesis that in TGR (hAGT)L1623 rats: 1) depressed cardiac contractility in LV myocytes is related to the upregulation of CaMKIIδ and β_3-AR combined with downregulation of β_1-AR; 2) CaMKII inhibition with KN-93, a cell-permeable and potent inhibitor of CaMKII [16,19,45,46] restores normal LV myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$, and β-adrenergic reserve.

2. Materials and methods

2.1. Animal model

This study was approved by the Wake Forest University School of Medicine Animal Care and Use Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 8th edition, updated 2011). Ten male Sprague Dawley (SD) and ten transgenic TGR (hAGT)L1623 hypertensive rats (5–6 -month-old; body weight, 450–600 g; without clinical indication of heart failure) were obtained from the Hypertension and Vascular Research Center animal colony at Wake Forest University School of Medicine. Animals were housed in the same environment and had free access to water and food.

2.2. Experimental protocol

Two sets of studies were conducted in Sprague Dawley (SD) and TGR (hAGT)L1623 male rats.

Functional Study: We assessed the cellular basis of LV dysfunction by measuring basal LV myocyte relaxation, contraction, and $[Ca^{2+}]_{iT}$ as well as the response to acute β-adrenergic stimulation by isoproterenol (ISO) in both SD and TGR (hAGT)L1623 rats. In TGR (hAGT)L1623 rats, these measurements were repeated after the pretreatment of myocytes with KN-93 or KN-92. In a subset of freshly isolated LV myocytes obtained from the same rats, we also determined myocytes β_3-ARs responsiveness.

Molecular Study: To investigate the molecular mechanism contributing to LV myocyte deficits, we determined myocyte CaMKIIδ protein levels, CaMKIIδ phosphorylation (pCaMKIIδ), and β_1-and β_3-AR expression in TGR (hAGT)L1623 rats.

2.3. Functional studies in isolated LV myocytes

2.3.1. Myocyte isolation

Using techniques well established in our laboratory, reproducible, high-yield (more than 85%), and calcium-tolerant cardiomyocytes were isolated from LV samples of TGR (hAGT)L1623 [47–49]. Briefly, experiments were performed in rats anesthetized with ketamine [12 mg/kg, i. p (Hospira, Inc., Lake Forest, Illinois).] and xylazine [1.8 mg/kg, i. p (Phoenix Pharmaceutical, Inc., Burlingame, CA). Animals were ventilated with a respirator (Model RSP1002, Kent Scientific Corp, Litchfield, CT) using oxygen-enriched room air with isoflurane (0.5–2%). The body temperature was maintained at 37 °C by a heating pad placed underneath the animal. Hearts, quickly removed through a splitting of the sternum, were weighed, and the isolated heart was perfused at 37 °C with a HEPES solution free of calcium (in mmol/L: HEPES 10.0, NaCl 110, MgSO4 1.2, D-mannitol 45.0, KH2PO4 1.2, glucose 15.0, and KCl 5.4) on a Langendorff apparatus. Five minutes after, the perfusion solution was changed to a calcium-HEPES-collagenase solution containing type II collagenase, 10 mg (0.04%, w/v) (290 U/mg, Worthington, Freehold, NJ), $CaCl_2$ 35 μmol/L, and bovine serum albumin 60 mg (0.1%, w/v) (Sigma-Aldrich Co. USA). The LV was removed from the apparatus and the flaccid heart and myocytes were immersed in sterilized centrifuge tubes containing a HEPES-collagenase solution. Spontaneous contractions of isolated myocytes were verified by *in-vivo* microscopy.

The isolated myocytes were centrifuged (at 4 °C, 500 rpm, for 1 min), the broken cells in the supernatant were removed. Then after each settling, the calcium concentrations were gradually increased in the HEPES solution from 250 μmol/L, 500 μmol/L, to 1000 μmol/L, respectively. Finally, the freshly isolated LV myocytes were kept in the study buffer (HEPES solution with 1.2 mM $CaCl_2$) at room temperature for a 2 h stabilization period· Then the isolated myocytes were counted, and the viability and morphology were examined. In each experiment, during the study period (10–14 h) rod-shaped cells were randomly selected for LV myocyte dimension measurements.

2.3.2. Evaluation of LV myocyte functional performance

2.3.2.1. LV myocyte basal contractile function and β-adrenergic reserve. As described by us previously [47], cardiomyocytes were loaded in dishes superfused with the study buffer. Field-stimulation at 0.5 Hz was applied to elicit cell contraction. Fluorescence and Contractility System (IonOptix, Milton, MA) was utilized for myocyte contraction and relaxation. After collecting baseline steady-state data, a non-selective β-AR agonist ISO (10^{-8} M) was perfused for 10 min. Data were continuously collected during ISO exposure for 10 min and 8 min after drug washout. LV myocyte β-adrenergic reserve was defined as the changes between baseline and post-superfusion functional responses.

2.3.2.2. Effects of a selective β_3-AR agonist stimulation on LV myocyte functional performance. Previously, we reported that HF is associated with opposing changes of cardiac β_1-and β_3-adrenergic signaling pathways. Myocardium β_3-AR are upregulated, but β_1-AR is down-regulated (unchanged β_2-AR expression) with resultant decreased β_1-AR-mediated positive inotropic action and an enhanced β_3-AR-coupled negative inotropic effect in the heart [50]. The restructuring of β-AR is an important cause of cardiac β-AR desensitization. To determine the contribution of the subtypes of β_3-AR stimulation on β-AR reserve in TGR (hAGT)L1623 hypertensive rats, the above protocol was repeated in subsets of myocytes following the addition of BRL-37,344 (BRL, 10^{-8} M) [35,37], a selective β_3-agonist. Data were collected for 10 min during drug exposure.

2.3.3. Simultaneous measurement of LV myocyte contractile and calcium transient responses

In a second series of experiments, different sets of LV myocytes were incubated with 10 mM indo-1-AM (Molecular Probes, Eugene, OR) and then placed in a flow-through T-culture dish. Myocytes' functional performance and calcium transient ($[Ca^{2+}]_{iT}$) responses were measured simultaneously with a dual-excitation fluorescence photo-multiplier system (IonOptix LLC, Westwood, MA) [37,51]. After stabilization, data were collected at baseline and after the superfusion of ISO or BRL. The absolute value of $[Ca^{2+}]_i$ is not reported because compartmentalization of loaded indo−1−AM into mitochondria is known to occur [37,52]. Therefore, we only report the ratio of the emitted fluorescence (410/490) to represent the relative changes in peak intracellular $[Ca^{2+}]_i$ before and after interventions.

2.3.4. Effects of CaMKII inhibition on myocyte function, β-AR reserve, and $[Ca^{2+}]_i$ regulation in TGR (hAGT)L1623

In a third series of experiments, the effects of CaMKII activation on LV myocyte function of TGR (hAGT)L1623 rats were determined. Briefly, different subsets of TGR (hAGT)L1623 myocytes were pretreated with a selective CaMKII inhibitor of KN-93 (10^{-6} M, 30 min) [16,53] or KN-92 (10^{-6} M, 30 min), an inactive KN-93 analog without CaM kinase inhibitory activity. The KN-92-treated myocytes were used as a negative control to rule out off-target effects [17,19]. Then the above protocols were repeated. Both contractile and $[Ca^{2+}]_i$ transient responses at baseline and followed by ISO (10^{-8} M) superfusion were recorded. As previously reported [35,47], the percent shortening (SA), the peak velocity of shortening (dL/dt_{max}), the peak velocity of re-lengthening (dR/dt_{max}) and the peak systolic $[Ca^{2+}]_{iT}$ were obtained.

2.4. Molecular studies in LV myocytes

To assess the molecular basis contributing to LV myocyte deficits in TGR (hAGT)L1623 rats, we measured LV myocyte CaMKIIδ protein levels, CaMKIIδ phosphorylation (at Thr287) (pCaMKIIδ) as well as the expressions of β_3-and β_1-ARs.

2.4.1. Determine CaMKIIδ protein levels and pCaMKIIδ expression

As previously described, isolated myocytes pre-chilled in Phosphate-Buffered Saline (PBS) were washed. Cellular protein was extracted in the presence of a protease and phosphatase inhibitor cocktail (ab201119, Abcam, Boston, MA) by Mammalian Protein Extraction Reagent (Thermo Fisher Scientific 78,501, Waltham, MA). The cell lysates (20 μg) were separated into 12% mini-protein gels (Bio-Rad, 456–1403, Hercules, CA), then blotted to a PVDF membrane (Thermo Fisher Scientific. 88,585, Waltham, MA). Primary antibodies employed in the study were CaMKII total (Abcam AB126789, Boston, MA), CaMKIIδ (Thermo Fisher Scientific, PA522168, Waltham, MA), and phosphorylated (T287) (Abcam AB182647, Boston, MA). All the band intensities were normalized to the internal loading control β-Actin [20].

2.4.2. Western blot analysis of β_1-, β_2-and β_3-AR

With pre-chilled PBS, cardiomyocytes were briefly washed before the addition of a membrane protein-extraction reagent (Pierce, Rockford, IL) with a proteinase inhibitor cocktail (Cell Signaling Technology, Danvers, MA) [35,54]**.** Cell membrane lysate (30 μg) was blotted to a PVDF membrane and then incubated with polyclonal IgG to β_1- β_2-and β_3-AR (1:1500 dilutions, Santa Cruz Biotechnology Inc. Santa Cruz, CA) at 4 °C overnight. Following washes, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit IgG (1:3000 dilutions, Sigma, St. Louis, MO). For normalization, the same blot was stripped and reprobed with polyclonal IgG to actin at 1:2500 dilutions (Santa Cruz Biotechnology, Inc.) [35]**.**

2.5. Statistical analysis

All data are expressed as mean ± standard deviation or mean ± standard error. Statistical comparisons among groups were made with repeated-measures ANOVA. Pairwise tests of individual group means were compared by the Bonferroni procedure. Measurements of myocyte contraction and $[Ca^{2+}]_{iT}$ were averaged from each animal and treated as a single data point. The mean differences in cell dynamics and indo-1-AM fluorescence ratios were calculated between groups. Significance was established as $P < 0.05$.

3. Results

3.1. Myocyte function, $[Ca^{2+}]_i$ regulation and acute CaMKII inhibition in TGR (hAGT)L1623

LV myocytes contraction, relaxation, and $[Ca^{2+}]_{iT}$ before and after ISO superfusion of SD control and TGR (hAGT)L1623 rats without and with pretreatment with KN-93 are summarized in Table 1, and exhibited in Fig. 1 and Fig. 3A. LV myocyte contractile

Table 1
Effects of KN-93 on myocyte basal and isoproterenol-stimulated functional performance and $[Ca^{2+}]_i$ transient in TGR(hAGT)L1623.

	SD Control (N = 10)		TGR (hAGT)L1623 (N = 10)		TGR (hAGT)L1623/KN-93 (N = 10)	
	Baseline	**Isoproterenol**	**Baseline**	**Isoproterenol**	**Baseline**	**Isoproterenol**
Resting length (μm)	113.3 ± 4.9	112.9 ± 4.5	126.8 ± 4.9	126.9 ± 5.6	126.8 ± 6.8	126.9 ± 5.9
Percent of shortening (SA, %)	10.0 ± 0.4	15.0 ± 0.6[c]	7.7 ± 0.4[a]	10.7 ± 0.56[c,d]	9.5 ± 0.6[b]	14.5 ± 0.9[c]
Velocity of shortening (μm/sec)	138.4 ± 6.1	224.5 ± 13.3[c]	104.9 ± 4.3[a]	150.3 ± 7.2[c,d]	134.4 ± 3.6[b]	215.0 ± 6.9[c]
Velocity of relengthening (μm/sec)	104.2 ± 3.5	154.8 ± 4.5[c]	92.7 ± 2.9[a]	122.9 ± 4.8[c,d]	107.4 ± 4.2[b]	161.1 ± 5.6[c]
Peak systolic $[Ca^{2+}]_i$ transient	0.20 ± 0.02	0.26 ± 0.02[c]	0.15 ± 0.01[a]	0.18 ± 0.02[c,d]	0.20 ± 0.01[b]	0.26 ± 0.01[c]

Values are mean ± standard deviation; N = number of rats.
SD: Sprague Dawley rats; TGR(hAGT)L1623: Transgenic rats expressing the human angiotensinogen gene in their genome; TGR(hAGT)L1623/KN-93: pretreatment of myocytes from transgenic rats with a CaMKII antagonist, KN-93.
[a] $p < 0.05$, TGR (hAGT)L1623 baseline vs. SD Control baseline.
[b] $p < 0.05$, TGR (hAGT)L1623/KN-93 baseline vs. TGR (hAGT)L1623 baseline.
[c] $p < 0.05$, Isoproterenol response vs. corresponding baseline value.
[d] $p < 0.05$, Isoproterenol-induced percent changes between TGR (hAGT)L1623 rats vs. SD Controls.

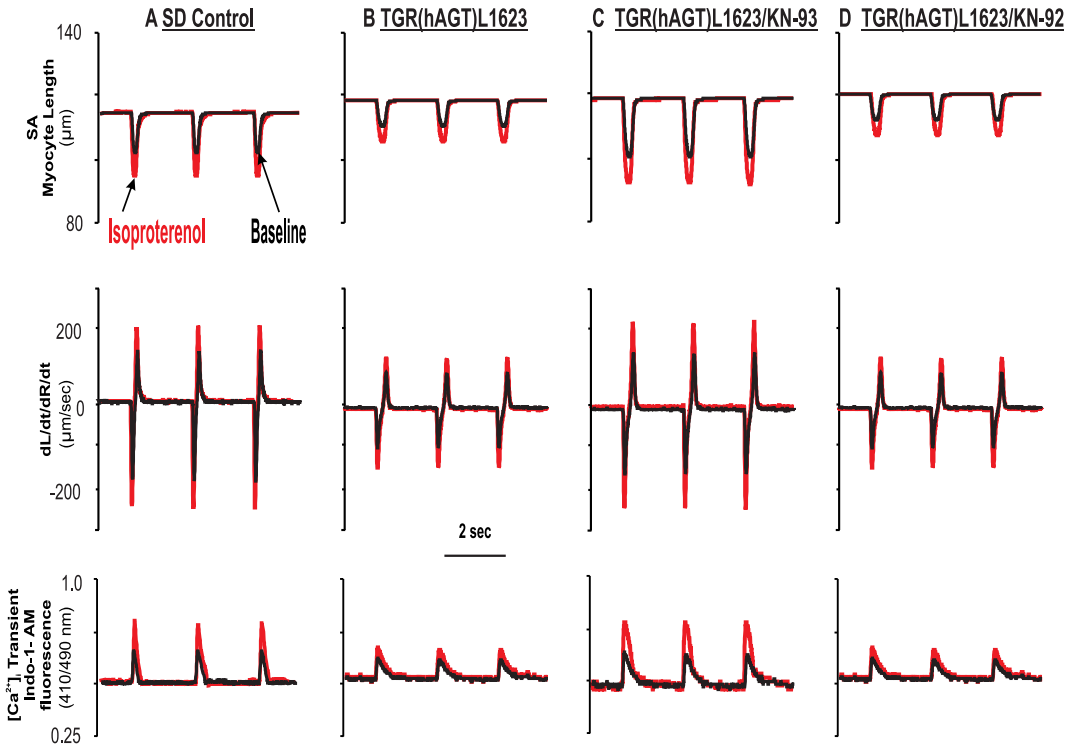

Fig. 1. Examples of TGR (hAGT)L1623 myocyte contractile dysfunction and impaired $[Ca^{2+}]_{iT}$ at baseline and in response to acute isoproterenol stimulation (β-adrenergic reserve). Representative of superimposed tracings recorded in electrically stimulated LV myocytes from: (A) one SD control myocyte; (B) one TGR (hAGT)L1623 myocyte; (C) one pretreated TGR (hAGT)L1623 myocyte with KN-93; and one pretreated TGR (hAGT) L1623 myocyte with KN-92 at baseline and after acute superfusion isoproterenol (ISO, 10^{-8} M). Shown are myocyte percent of shortening (SA), peak velocity of shortening (dL/dt_{max}) and re-lengthening (dR/dt_{max}), and the peak $[Ca^{2+}]_{iT}$.

functional and $[Ca^{2+}]_{iT}$ responses to a selective β3-AR agonist stimulation of SD and TGR (hAGT)L1623 rats are displayed in Fig. 2 and summarized in Table 2 and Fig. 3B.

3.1.1. Effects of KN-93 on myocyte functional responses at baseline in TGR (hAGT)L1623

Versus SD control (Fig. 1A), TGR (hAGT)L1623 myocytes had significantly impaired contractile function and $[Ca^{2+}]_{iT}$ (Tables 1 and 2, Fig. 1A and B). As summarized in Table 1, compared with SD controls, in TGR (hAGT)L1623 rats, LV myocyte lengths increased significantly (126.8 ± 4.6 vs 113.3 ± 4.9 μm). There were significant decreases in cell systolic amplitude, dL/dt_{max} (104.9 ± 4.3 vs 138.4 ± 6.1 μm/s) and dR/dt_{max} (92.7 ± 2.9 vs 104.2 ± 3.5 μm/s). $[Ca^{2+}]_{iT}$ (0.20 ± 0.02 vs 0.15 ± 0.01) was also significantly decreased by 25% with slowed the decline of $[Ca^{2+}]_i$.

Of importance, pretreatment of cardiac myocytes with KN-93 restored myocyte normal basal contraction (dL/dt_{max},134.4 ± 3.6

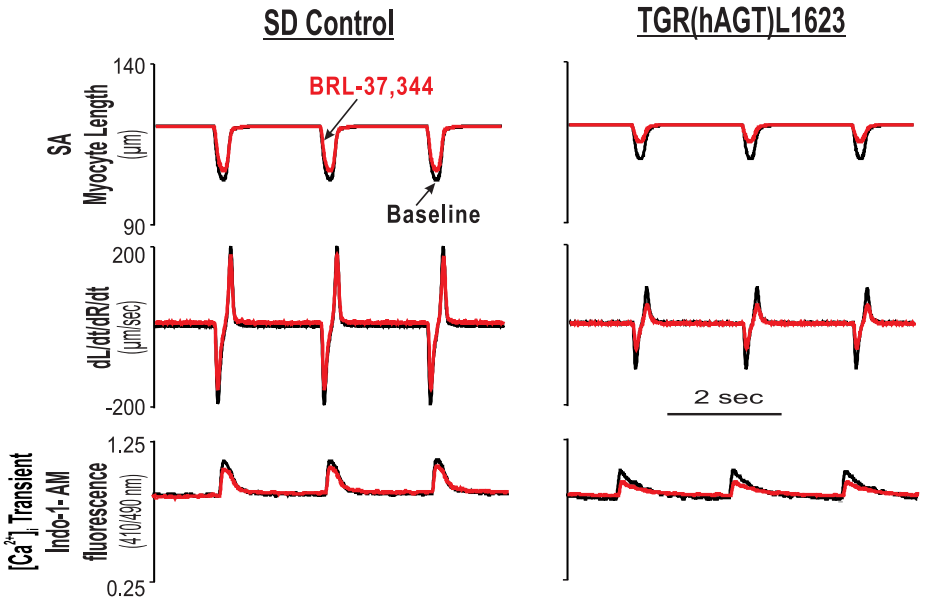

Fig. 2. Examples of acute β_3-AR stimulation with BRL-37,344 produced enhanced negative modulation on contractile function and $[Ca^{2+}]_{iT}$ inTGR (hAGT)L1623 myocyte. Representative of superimposed tracings recorded in LV myocytes from one SD control myocyte and one TGR (hAGT)L1623 myocyte at baseline and during BRL-37,344 superfusion.

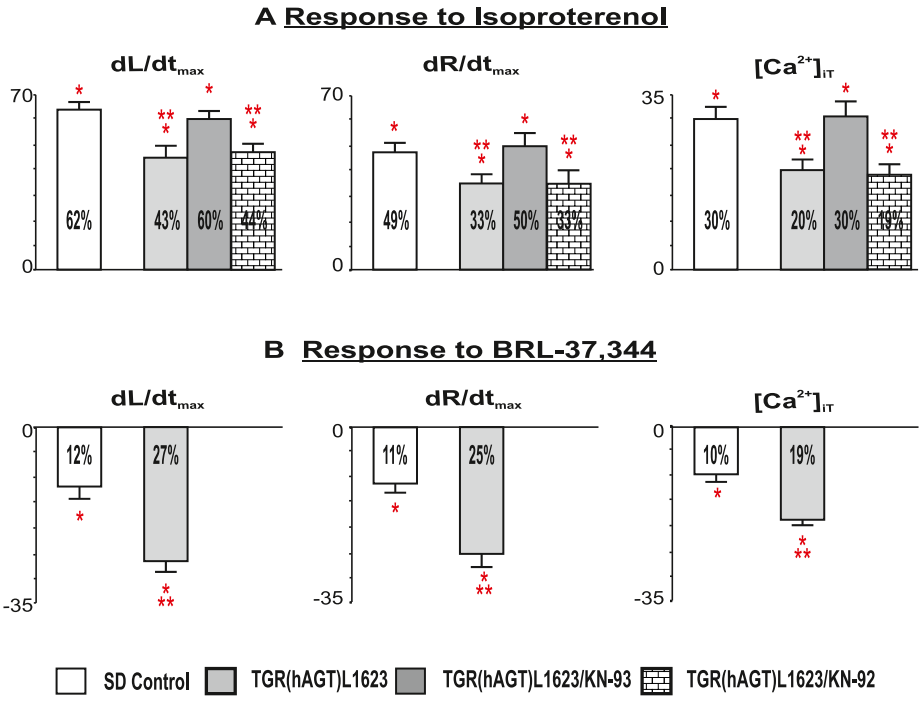

Fig. 3. (A)Pre-treatment with KN-93 restores normal myocyte contractile functional and $[Ca^{2+}]_{iT}$ responses to isoproterenol stimulation in TGR (hAGT)L1623 rats. Group means of LV myocytes obtained from SD control group (N = 10), TGR (hAGT)L1623 group (N = 10), and TGR (hAGT) L1623 myocytes pretreated with KN-93 (N = 8) or TGR (hAGT)L1623 myocytes pretreated with KN-92 (N = 5). N, Numbers of rats. $*p < 0.05$, isoproterenol response vs. corresponding baseline value. $**p < 0.05$, Percent changes of TGR (hAGT)L1623 myocytes without or with pretreatment of KN-92 vs SD control myocytes. (B) Enhanced inhibition of myocyte contractile function and $[Ca^{2+}]_{iT}$ to BRL-37,344 stimulation in TGR (hAGT) L1623 rats. Group means (± standard deviation) of LV myocytes were obtained from SD control group (N = 8) and TGR (hAGT)L1623 group (N = 10) and TGR (hAGT)L1623 group pretreated with KN-93 (N = 8) or KN-92 (N = 5). $*p < 0.05$, BRL-37,344 response vs. corresponding baseline value. $**p < 0.05$, TGR (hAGT)L1623 vs SD control myocytes.

Table 2
Myocyte Functional Performance and $[Ca^{2+}]_i$ Transient to β_3-AR Stimulation in TGR (hAGT)L1623.

	SD Control (N = 8)		TGR (hAGT)L1623 (N = 8)	
	Baseline	BRL-37,344	Baseline	BRL-373,44
Resting length (μm)	113.0 + 6.5	112.9 + 6.2	127.0 + 6.7[a]	126.8 + 6.5127.8
Percent shortening (SA; %)	10.0 + 1.5	8.7 + 1.1[b]	7.5 + 1.0[a]	5.3 + 0.8[b,c]
Velocity of shortening (μm/sec)	137.6 + 8.7	121.0 + 6.6[b]	101.3 + 10.5[a]	74.4 + 4.5[b,c]
Velocity of relengthening (μm/sec)	104.0 + 7.7	92.7 + 6.4[b]	88.9 + 4.6[a]	66.4 + 3.7[b,c]
Peak systolic $[Ca^{2+}]_i$ transient	0.21 + 0.02	0.19 + 0.01[b]	0.16 + 0.01[a]	0.13 + 0.01[b,c]

Values are mean ± standard deviation; N = number of rats.
SD: Sprague Dawley rats.
TGR(hAGT)L1623: Transgenic rats expressing the human angiotensinogen gene in their genome.
[a] $p < 0.05$, TGR (hAGT)L1623 baseline vs. SD Control baseline.
[b] $p < 0.05$, BRL-37,344 response vs. corresponding baseline value.
[c] $p < 0.05$, BRL-37,344-induced percent changes between TGR (hAGT)L1623 rats vs. SD Controls.

μm/s), relaxation (dR/dt_{max}, 107.4 ± 4.2 μm/s) and $[Ca^{2+}]_{iT}$ (0.20 ± 0.01) to values similar to those determined in SD rats (Table 1, Figs. 1C and 3A). By contrary, compared with SD control myocytes, in TGR (hAGT)L1623 myocytes-pretreated with KN-92, impaired basal cell function persisted with significant decreases in SA (7.6 ± 1.4 vs 10.2 ± 0.9%), dL/dt_{max} (106.3 ± 10.8 μm/s), dR/dt_{max} (90.2 ± 1.6 μm/s) and $[Ca^{2+}]_{iT}$ (0.16 ± 0.01) (Figs. 1D and 3A).

3.1.2. *Effects of KN-93 on myocyte functional responses to β-AR stimulation in TGR (hAGT)L1623*

Versus SD, TGR (hAGT)L1623 LV myocytes had significantly attenuated β-AR agonist-mediated positive modulation. As summarized in Table 1 and displayed in Figures 1 A and B, in SD myocytes compared with baseline, in response to acute ISO stimulation, dL/dt_{max}, dR/dt_{max}, and $[Ca^{2+}]_{iT}$ were significantly increased by 62%, 49%, and 30% respectively. On the other hand, in TGR (hAGT) L1623 myocytes, ISO produced significantly lower increases in dL/dt_{max} (43%), dR/dt_{max} (33%), and $[Ca^{2+}]_{iT}$ (20%), indicating a significantly reduced β-AR reserve. Of importance, in TGR (hAGT)L1623 myocytes previously exposed to KN-93, ISO-caused increases in dL/dt_{max} (60%), dR/dt_{max} (50%), and $[Ca^{2+}]_{iT}$ (31%) were also significantly augmented and were also close to SD control values, demonstrating restoration of cardiac β-AR normal responsiveness (Fig. 3A).

3.1.3. *Effects of KN-92 on myocyte functional responses in TGR (hAGT)L1623*

Compared with SD control myocytes (Figs. 1 and 3A), in TGR (hAGT)L1623 myocytes-pretreated with KN-92, impaired basal and β-AR stimulated cell functional responses persisted with significant decreases in basal SA (7.5 ± 0.1%), dL/dt_{max} (101.7 ± 3.3 μm/s), dR/dt_{max} (90.8 ± 3.6 μm/s) and $[Ca^{2+}]_{iT}$ (0.16 ± 0.01). Acute ISO infusion also produced significantly less increases in SA (28%, 9.7 ± 0.9 vs 7.6 ± 2.4%), dL/dt_{max} (44%, 146.5 ± 6.7 vs 101.7 ± 3.3 μm/s), dR/dt_{max} (33%, 120.7 ± 3.1 vs 90.8 ± 3.6 μm/s), and $[Ca^{2+}]_{iT}$ (19%, 0.19 ± 0.01 vs 0.16 ± 0.01), demonstrating a decline in β-adrenergic reserve (Figs. 1 and 3A).

3.1.4. *Effects of β_3-AR stimulation on cardiomyocyte functional performance*

As shown in Table 2 and exhibited in Figs. 2 and 3B, versus SD, in TGR (hAGT)L1623 cardiomyocytes, BRL-37,344 (10^{-8} M) caused significantly greater decreased SA (29% vs 13%), dL/dt_{max} (27% vs 11%), dR/dt_{max} (25% vs. 11%) and $[Ca^{2+}]_{iT}$ (19% vs. 10%) (Fig. 3B), demonstrating enhanced β_3-AR-mediated negative modulation on TGR (hAGT)L1623 LV myocyte function and $[Ca^{2+}]_i$ regulation.

3.2. *Molecular basis of cardiomyocyte defects in TGR (hAGT) L1623 rats*

TGR (hAGT)L1623-induced alterations of LV myocyte CaMKIIδ protein levels, pCaMKIIδ, as well as β_1-and β_3-AR expressions, are displayed in Figs. 4 and 5.

3.2.1. *Myocyte CaMKIIδ protein levels and CaMKIIδ phosphorylation (at Thr287)*

As shown in Fig. 4, in TGR (hAGT)L1623 myocytes, compared with SD control myocytes, the total CaMKII (CaMKII) was relatively unchanged in (Fig. 4A). The CaMKIIδ protein levels (16%, 0.50 ± 0.04 vs. 0.43 ± 0.02) were not significantly increased. While at the same time, the signal ratio of the phosphorylated form of CaMKIIδ protein to β-Actin was significantly increased by 35% (0.27 ± 0.01 vs. 0.20 ± 0.01) (Fig. 4B) in myocytes from TGR (hAGT)L1623 rats, indicating increased LV CaMKIIδ activation.

3.2.2. *Myocyte subtype β-AR expression*

As shown in Fig. 5, Western blot analyses were performed to quantify myocyte β_1-and β_3-AR protein levels in SD control and TGR (hAGT)L1623 rats. Fig. 5A displays the examples of β_1-AR and β_3-AR expressions in LV myocytes from one SD control and one TGR (hAGT)L1623 rat. β-Actin was reprobed as a loading control. Fig. 5B presents the group means of LV myocyte β_1-AR and β_3-AR protein levels in both SD control and TGR (hAGT)L1623 rats. Versus SD, in TGR (hAGT)L1623 rats, there were significantly decreased LV myocytes β_1-AR protein levels. The signal ratio of β_1-AR protein to β-actin was decreased by 28% (0.39 ± 0.04 vs. 0.54 ± 0.02). While

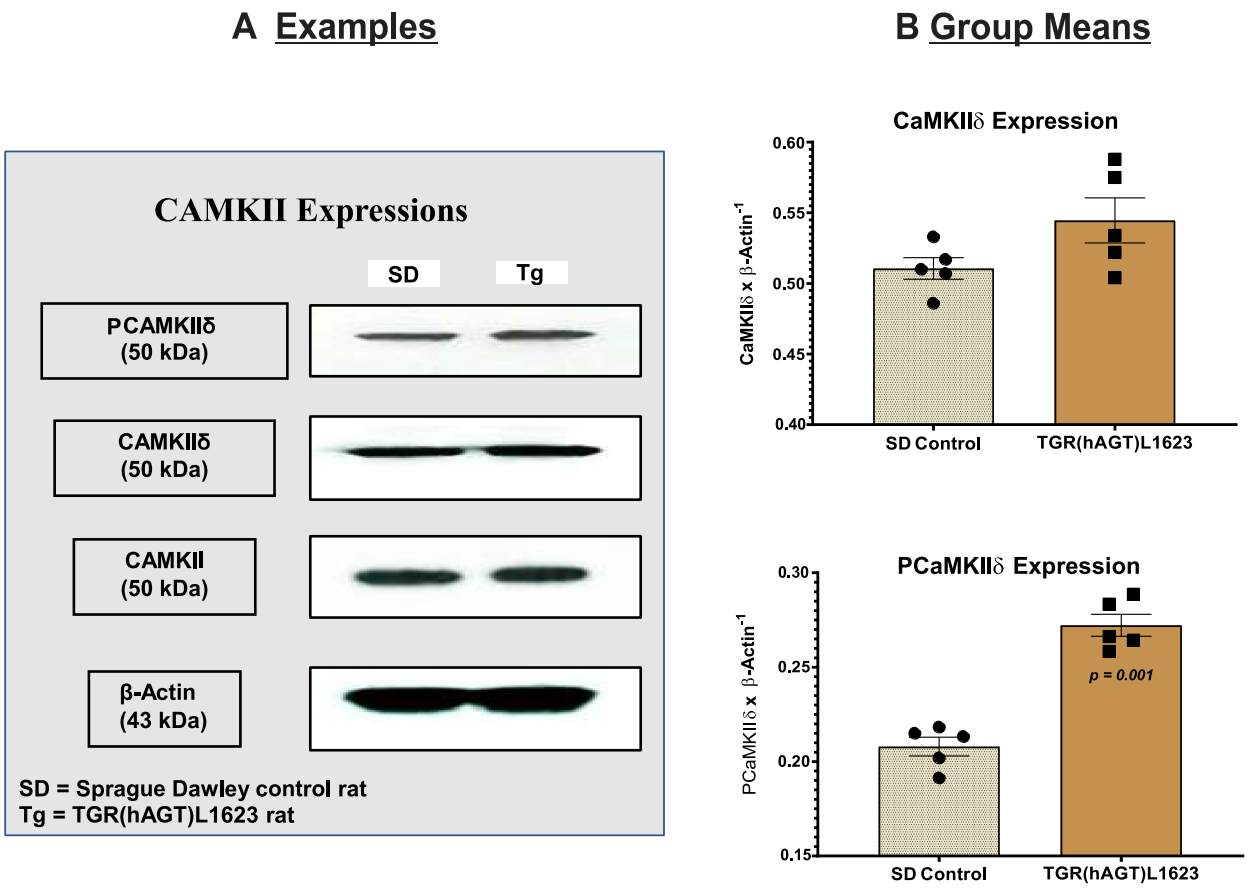

Fig. 4. LV myocyte expression and activity of CaMKIIδ in SD control and TGR (hAGT)L1623 rats. A: Examples of Thr287 phosphorylated CaMKII δ (PCaMKII δ), CaMKIIδ and total CaMKII (CaMKII) (CaMKII) in LV myocytes from one SD control and one TGR (hAGT)L1623 rat. β-Actin was reprobed as a loading control. B: Bar graphs are group means (± standard error) (N = 5 per group) of CaMKIIδ and PCaMKIIδ protein levels in myocytes from control and transgenic rats. *p < 0.05, TGR (hAGT)L1623 vs SD control.

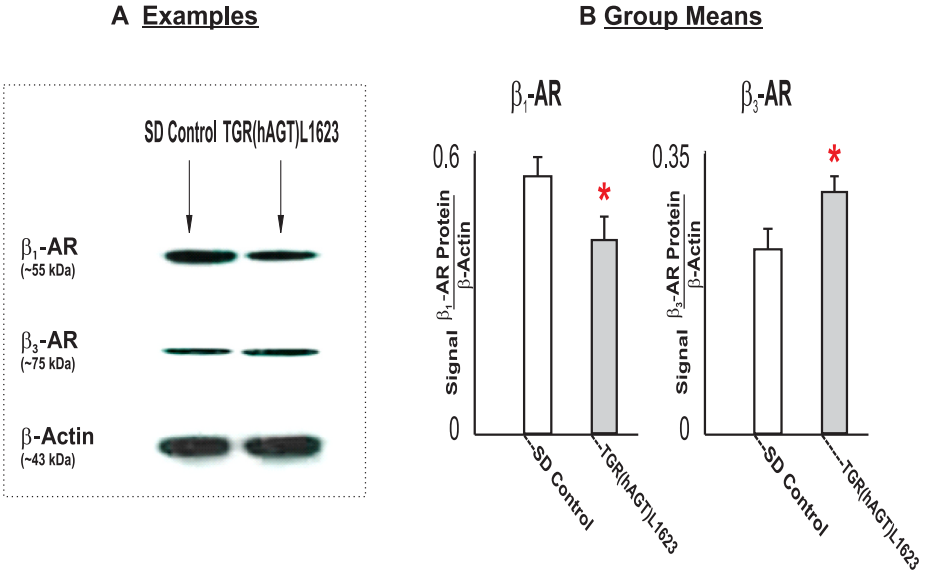

Fig. 5. LV myocyte β1-AR and β3-AR expressions in SD control and TGR (hAGT)L1623 rats. A: Examples of β1-AR and β3-AR expressions in LV myocytes from one SD control and one TGR (hAGT)L1623 rat. β-Actin was reprobed as a loading control. B: Bar graphs are group means (± standard error) (N = 6 per group) of β1−AR and β3-AR protein levels in myocytes from SD control and TGR (hAGT)L1623 rats. *p < 0.05, TGR (hAGT)L1623 vs SD control.

the rations of β_3-AR protein to β-actin were significantly increased by 30% (0.30 ± 0.02 vs. 0.23 ± 0.01) in TGR (hAGT)L1623 myocytes.

4. Discussion

We examined the cellular and molecular mechanisms of declines in cardiac contractile function and β-AR reserve of a newly established humanized model of hypertension in transgenic rats expressing the human AGT gene. Our study demonstrates, for the first time: 1) TGR (hAGT)L1623 rats have defects in cardiomyocyte contraction, relaxation, and $[Ca^{2+}]_i$ regulation with diminished β-AR inotropic reserve; 2) expression of the human AGT gene in TGR (hAGT)L1623 augments LV myocytes CaMKIIδ activation, with upregulation of β_3-AR, but down-regulation β_1-AR, independent of HF; 3) the increase in cardiac CaMKIIδ activation exacerbates the impairments of $[Ca^{2+}]_i$ regulation and β-adrenergic modulation, enhancing the inhibition of LV myocyte contraction and relaxation; 4) pretreatment with the CaMKII inhibitor KN-93 restores normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$ and β-adrenergic reserve in TGR (hAGT)L1623.· These findings indicate that the defect in cardiomyocyte force-generating capacity and relaxation process with impaired $[Ca^{2+}]_i$ regulation and increased CaMKIIδ activation are critical elements promoting cardiac dysfunction in this humanized hypertension model. These findings extend our understanding of the role of CaMKIIδ in the regulation of the heart function in the context of hypertension and suggest a novel therapeutic potential for cardiac CaMKIIδ inhibition to reverse cardiac dysfunction in cardiac hypertrophy and hypertension. The data reported here provides the preclinical data sustaining the initiation of therapeutic trials based on the inhibition of CaMKIIδ.

4.1. Cardiomyocyte functional impairment and $[Ca^{2+}]i$ dysregulation in TGR (hAGT)L1623 rats

There is abundant evidence for a tissue role of Ang II in the evolution of heart failure [55]. Ang II concentrations in cardiac tissue are independently regulated from the circulation [56–58]. Intracrine Ang II generation [59–61] remains unchanged in rodents' hearts during exposure to systemic administration of angiotensin-converting enzyme (ACE) inhibitors [62,63] or Ang II receptor blockers (ARBs) [64,65]. Previously, we [6,13] and others [26] performed serial studies in this humanized model of hypertension and reported collective characterization of the hemodynamic, transthoracic echocardiography, biochemical, and RAS profile [5,6]. We showed that rats expressing the human AGT gene exhibit sustained hypertension, cardiac hypertrophy, and systolic dysfunction. These hypertensive rats had increased heart rate lability associated with increased neurogenic activity. TGR (hAGT)L1623 rats had significantly reduced velocity of circumferential shortening of Vcf and peak early filling velocity of E. These changes occurred in the absence of alteration in FS, fractional shortening.

This model constitutes a high Ang II type hypertension in which the blood pressure elevation is associated with a 4-fold increase in LV Ang II content [13] and a blunted effect of Ang II to augment the higher baseline values of L-type Ca^{2+} current ($I_{Ca,\ L}$) density [15]. Furthermore, a14−day therapy with valsartan normalized the blood pressure of TGR (hAGT)L1623 rats without suppressing the high cardiac Ang II content [6].

In extending previous findings from our laboratory, we confirm that at baseline, compared with SD, in the freshly isolated LV myocytes from TGR (hAGT)L1623 rats there were about 23%, 24% and 11% decreases in the percent shortening (SA), myocyte contractility (dL/dt_{max}) and relaxation rate (dR/dt_{max}), respectively. There was significantly decreased $[Ca^{2+}]_{iT}$ with markedly elevated diastolic $[Ca^{2+}]_i$. Importantly, versus SD, in TGR (hAGT)L1623 LV myocytes, ISO, a β-AR agonist caused positive modulation on myocyte contraction, relaxation, and $[Ca^{2+}]_{iT}$ was significantly attenuated (Table 1). It is noted that we observed that the reduced LV myocyte contractile function in TGR (hAGT)L1623 rats occurred in the absence of HF. This is consistent with our past report that these TGR (hAGT)L1623 rats have cardiac hypertrophy with preserved ejection fraction (EF%) and fractional shortening (FS%). There is no clinical indication of HF. LV myocyte SA and dL/dt_{max} were used as a measure of contractility because they removed the effects of extracardiac factors influencing contractility. The indices employed in the current experiments provided a more sensitive means of evaluating cardiac contractility. In fact, the amplitude of reductions in SA and dL/dt_{max} we demonstrated here were significantly smaller than we and others reported in various experimental models and humans with HF [29,39,47].

The observed LV contractile dysfunction with TGR (hAGT)L1623 rats suggests that the defects in $[Ca^{2+}]_i$ regulation may be the primary driver of the altered cardiomyocyte force-generating capacity and relaxation of this model with hypertension-induced hypertensive cardiomyopathy. These findings agree with a previous demonstration of impaired L-type calcium currents in myocytes from TGR (hAGT)L1623 rats and the downregulation of cardiac PI3-kinase and Akt in transgenic mice with cardiac-specific expression of a transgene fusion protein releasing Ang II from cardiomyocytes [55].

4.2. Molecular basis of cardiomyocyte defects in TGR (hAGT) L1623 rats

Pressure-overload hypertrophy and hypertrophic cardiomyopathy signaling are complex with several well-defined molecular players contributing to ventricular dysfunction. These proteins function in intracellular calcium cycling and thereby regulate systolic and diastolic function characteristics of hypertrophic phenotype and HF phenotype. The multifunctional serine/threonine kinase, CaMKII is a nodal point in the regulation of intracellular Ca^{2+} handling, ion channels, and gene transcription [18,27,66–68]. CaMKII has both short-term signaling effects in maintaining excitation–contraction coupling and long-term effects on gene transcription in cardiomyocytes [69]. Activation of the multifunctional kinase CaMKIIδ has emerged as a key nodal point in the translation of cellular stresses into downstream alterations to cardiac physiology [16,20,29,70]. Although others have suggested that CaMKII plays a major role in the development of HF induced by stresses including pressure overload or angiotensin II infusions [19,22–24,30], the

physiological role of CaMKII remained undefined. This problem is aggravated by a literature reporting both detrimental [20,24,25] or beneficial [19,26–30] effects of CaMKII inhibition. In the current study, we found that in LV myocytes from TGR (hAGT)L1623 rats, there was no significant difference in total CaMKIIδ expression, but pCaMKIIδ was significantly increased, indicating increased CaMKIIδ activation. It is evident that many mechanisms contribute to the excessive activation of CaMKII in cardiac hypertrophy. For example, oxidative stress and Ca^{2+} overload may activate CaMKII by Ca^{2+}/CaM-dependent and oxidation-dependent pathways [71]. Like our finding, compared to lean controls, total CaMKIIδ expression was unchanged, but pCaMKIIδ was significantly increased in the diabetes mellitus Zucker Diabetic Fatty rats [20]. Hypertrophied myocardium from spontaneously hypertensive rats also showed increased CaMKII activity [72] without significantly increased expression of CaMKIIδ. Notably, these changes in CaMKII could be reversed by ACE inhibition, which also led to complete regression of the myocardial hypertrophy (presumably Ang II-mediated) that developed in this model [73]. These results suggest that the increased phosphorylation of cardiac CaMKIIδ, not the expression of total CaMKIIδ, could play a vital role in cardiac hypertrophy.

One of the most widely acknowledged roles of CaMKIIδ in the heart is the modulation of Ca^{2+} flux [20,29,69,74]. CaMKIIδ regulates proteins associated with cardiac Ca^{2+} flux, including ryanodine receptors, phospholamban, the SR Ca^{2+}-ATPase (SERCA2a), and L-type Ca^{2+} channels. Consistently, our data indicate that the differences in contractile performance between SD and TGR (hAGT) L1623 myocytes, as well as the restoration of contractility by CaMKIIδ inhibition with KN-93, are derived from alterations to Ca^{2+} transients and intracellular Ca^{2+} load. We found that KN-93, but not KN-92 normalized TGR (hAGT)L1623 myocyte systolic $[Ca^{2+}]_{iT}$ and diastolic intracellular Ca^{2+} levels at baseline and response to ISO stimulation, thereby fully restoring basal and β-AR-stimulated LV myocyte contractility and relaxation in TGR (hAGT)L1623 rats. Our findings are different than the study by Daniels et al. [20], who reported that CaMKIIδ inhibition attenuated the reduced force development and impaired rates of contraction and relaxation associated with type 2 diabetes, but these effects are independent of Ca^{2+} flux properties. Our results also differ from a previous study reporting that chronic KN-93 treatment in pressure-overload HF (15 days after transverse aortic constriction) reversed systolic dysfunction and diminished cardiac reserve without improvement of LV diastolic function [19]. Moreover, contrary to our findings, Baier et al. [24] observed adverse effects of CaMKII inhibition. They reported that CaMKII activation significantly increased in WT TAC vs. sham mice 2 days post-TAC. Cardiac dysfunction due to CaMKII inhibition is a potential cause of increased mortality in AC3-I TAC mice. Factors contributing to these conflicting and inconsistent findings include differences in animal models and procedures used to alter cardiac function and contractility. It is likely that the very different observations in CaMKII inhibition-mediated functional responses are mainly owing to using animal models with distinct cardiac pathophysiology. Further in-depth investigations of different animal models with human diseases are of vital importance. Our findings are supported by previous studies using animal models with global deletion of CaMKIIδ, which have reported protection against cardiac hypertrophy [19], apoptosis, and Ca^{2+} leak-induced arrhythmia [28]. Pharmacological inhibition of CaMKIIδ has also shown promising results both in vitro and in vivo [29]. Acute CaMKIIδ inhibition in isolated cardiac muscle taken from patients with ischemic HF resulted in increased contractility and reduced Ca^{2+} spark frequency [30].

The physiological role of CaMKII is not well defined. CaMKIIδ has been reported variably to have a negative inotropic effect [25], or positive inotropic effect [26,27] on normal LV contractile performance. Kaurstad et al. [26] reported that CaMKIIδ activation has a positive modulation of inotropy in the healthy heart. This interpretation is based on the finding that CaMKIIδ inhibition disrupts the beneficial effects of exercise on cardiac function in the healthy myocardium. Kemi et al. [27] suggested that the improved inotropy and lusitropy they observed in cardiomyocytes after exercise training in healthy mice was due to an increased CaMKIIδ activation. Apparently, as shown in the present study, the positive role of CaMKIIδ in the healthy heart is reversed to a key pathological player during chronic cardiac stress as occurs in, for example, hypertension.

Multiple mechanisms contribute to the excessive activation of cardiac CaMKII in hypertension. For example, Ca^{2+} overload and oxidative stress, the known features of cardiac hypertrophy, activate CaMKII via Ca^{2+}/CaM-dependent and oxidation-dependent pathways [71]. CaMKII activation also can occur downstream of neurohormonal stimulation. For instance, in the current study, increased Ang II and sympathetic nervous system (SNS) stimulation may promote cardiac CaMKII overactivation in TGR (hAGT)L1623 rats. The rescued myocyte function we observed in KN-93 pretreated TGR (hAGT)L1623 myocytes may be due to KN-93 antagonist action on the CaMKIIδ activation-induced adverse function effect, or might result from KN-93 preventing the increased CaMKIIδ activation in TGR (hAGT)L1623 myocytes. This interpretation is in keeping with the finding that KN-93 significantly inhibited the phosphorylation of CaMKII at Thr287 [72].

Importantly, we now report impaired LV myocyte $[Ca^{2+}]_i$ regulation at baseline as well as after β-AR stimulation in TGR (hAGT) L1623 rats. We found that in TGR (hAGT)L1623 myocytes, there are significantly reduced β_1-AR protein levels with unchanged β_2-AR protein levels (data not shown), but significantly increased β_3-AR expression with enhanced negative modulation on myocyte contraction, relaxation, and $[Ca^{2+}]_{iT}$ after BRL-37,344 superfusion. The upregulation cardiac β_3-AR-coupled pathway may be caused by activation of SNS in this model. LV myocyte CaMKIIδ overactivation with associated contrast changes in β_3-AR and β_1-AR may be an important cause of the abnormal contractile phenotype and β-AR desensitization in this humanized hypertension model of TGR (hAGT) L1623. Moreover, CaMKII inhibition with KN-93 completely restores normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$ and β-adrenergic reserve in TGR (hAGT)L1623. These findings indicate an important interplay between β3-AR and CaMKII and support the view that a significant crosstalk exists between β-AR signaling and CaMKII activation. CaMKII may serve as a downstream mediator of detrimental β_3-AR signaling in the abnormal contractile phenotype of cardiac hypertrophy and HF [43,75]. The mechanisms linking β_3-AR to CaMKII are unclear. Further in-depth investigations are clearly needed. Of note, a special emphasis needs to be made on the current limitations for cardiovascular disease (including HF and hypertension) therapies. It has been reported that ACE inhibitors or even ARBs have limited efficacy in reversing or halting the progression of cardiovascular disease [76–78]. Treatment with β-AR blockers is unexpectedly not associated with a modulation of CaMKII. CaMKII is not effectively targeted by current therapeutic

approaches in HF [79]. Therefore, directly targeting CaMKII signaling may have a future in cardiac translational therapy.

4.3. Study limitations

Our study has some limitations. Although KN-93 is the most widely used inhibitor to study cellular and in vivo functions of CaMKII [16], the drug inhibits L-type Ca^{2+} current ($I_{Ca,L}$) and voltage-dependent K^+ (K_v), which may be independent of CaMKII actions [17, 80]. On the other hand, KN-92 has similar blockade effects on $I_{Ca,L}$, and K_v at the concentration used to inhibit CaMKII [80–82]. Comparing the effects of KN-93 and KN-92 appears to address the most known off-target effects of KN-93 [17,19,82]. However, the effects of KN-93 in hypertension may also be due to some currently unknown molecular effects. Second, KN-93 blocks only the non-phosphorylated CaMKII acting as an allosteric inhibitor. It does not block the catalytic activity of the enzyme, which may be a more effective approach because the autonomous activity is resistant to allosteric inhibition caused by KN-93 [83]. Finally, we do not know whether the same effects may be duplicated when the inhibitor is given chronically. Future studies will address these possibilities.

5. Conclusions

Our study demonstrates, for the first time, that increased expression of plasma and cardiac AGT produces LV myocyte CaMKIIδ overactivation with associated contrasting changes in β_3-AR and β_1-AR followed by intrinsic defects in the cardiomyocyte force-generating capacity and relaxation process with impaired $[Ca^{2+}]_i$ regulation and decreased β-adrenergic reserve. Acute CaMKII antagonist with KN-93 restores normal intrinsic myocyte contraction, relaxation, $[Ca^{2+}]_{iT}$, and β-adrenergic reserve in the hypertension model. Our current findings and past reports [21] support the view that CaMKIIδ may play an integrative but unrecognized role in hypertension. These data provide new insights and strong evidence that excessive CaMKIIδ plays a vital role in maladaptive hypertrophy and hypertension and suggests that cardiac CaMKII inhibition may be a promising therapeutic approach to combat the development of hypertrophy and HF during chronic hypertension.

Author contribution statement

Xiaoqiang Sun, Jing Cao, Heng Jie Cheng: Conceived and designed the experiments; Performed experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools and data; Wrote the paper.

Zhe Chen, Yixi Liu: Performed experiments; Analyzed and interpreted the data.

Jessica L VonCannon: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools and data; Wrote the paper.

Carlos M Ferrario, Che Ping Cheng: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Ethics statement

This study was approved by the Wake Forest University School of Medicine Animal Care and Use Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 8th edition, updated 2011).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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