CALL FOR PAPERS | Mechanisms of Diastolic Dysfunction in Cardiovascular Disease

Renin overexpression leads to increased titin-based stiffness contributing to diastolic dysfunction in hypertensive mRen2 rats

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NEW & NOTEWORTHY

It is shown that activation of the renin-angiotensin-aldosterone system leads to hypertension and impaired cardiac relaxation associated with increased cardiomyocyte stiffness and hyperphosphorylation of the PEVK region of titin. Moreover, diastolic dysfunction in mRen2 rats occurs without changes in systolic parameters, similarly to human heart failure with preserved ejection fraction.

HYPERTENSION (HTN) AS A HUMAN EPIDEMIC (25) is considered as a major cardiovascular risk factor (33) in particular for heart failure (HF). The effectiveness of renin-angiotensin-aldosterone (RAAS) inhibitors in HTN therapy indicates a crucial role of RAAS in the pathomechanism of HTN (38).

Indeed, RAAS can be unfavorably activated upon the cardiovascular disease continuum (13), inducing and maintaining HTN, and thereby promoting HF (10). Nonetheless, the HF phenotype implies distinct entities (6), since the pathomechanism of HF with preserved ejection fraction (HFrEF) is different from that of HF with reduced ejection fraction (HFrEF) in human, representing a major clinical challenge (5). In particular, HTN is the most common comorbidity associated with HFrEF (10), but the clinical effectiveness of RAAS inhibitors in treating HFrEF is controversial (5–6, 44), in contrast with their beneficial effects in HFrEF (5–6, 13). This warrants an in-depth evaluation of RAAS in HFrEF.

The effects of RAAS activation can be studied in various animal models, including the transgenic rat strain carrying the mouse Ren-2 renin gene (mRen2), in which the angiotensin II (ANG II)-dependent primary HTN is present in a precisely defined genetic background (29, 34). Noteworthy is that the mRen2 phenotype is similar to those observed in human HF (5–6, 10, 13, 25, 38).

To this end, we aimed to investigate the influence of a well-defined genetic variation of RAAS on overall cardiac performance of mRen2 animals in detail. Blood pressure (BP) and cardiac contractility were measured by tail-cuff BP assessment and echocardiography in vivo repeatedly for 4 wk. Thereafter, cardiac morphology was widely investigated by different histological techniques. Mechanisms of cardiac...
function in transgenic animals were addressed in vitro by measuring Ca\(^{2+}\)-dependent active and titin-based passive tension of isolated cardiomyocytes from 15-wk-old male homozygous mRen2 rats. Finally, we performed biochemical assays to relate molecular changes to the mechanical alterations.

In summary, the purpose of our study was to determine how activation of RAAS affects myocardial function and to identify the underlying molecular mechanisms.

**MATERIALS AND METHODS**

**Animal experiments.** All animal care and experimental procedures were approved by the Ethical Committee of the University of Debrecen (Ethical Statement No. 1/2013/DE MÁB) and conformed to Directive 2010/63/EU of the European Parliament. Male homozygous rats carrying the mouse Ren-2 renin gene (mRen2, \(n = 6\)) were obtained from the Max Delbrück Center Für Molekulare Medizin [Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin-Buch, Germany]. Transgenic mRen2 rats were compared with age-matched male Sprague-Dawley rats (SD, \(n = 6\); Wobe, Budapest, Hungary). Animals were delivered to our laboratory at 9 wk of age. Experiments were carried out on 11- to 15-wk-old rats. No medication (e.g., antihypertensive drug) was administered to study subjects, and animals were fed a standard chow and tap water ad libitum. BP and heart rate (HR) were monitored by noninvasive CODA tail-cuff method (Kent Scientific, Torrington, CT). The average of five successful measurements on each animal was used to obtain single BP values. Body temperature was 37 ± 0.5°C upon measurements. The body weight (BW) of the animals was measured at 15 wk of age, and then the animals were killed. Following blood collection by inferior vena cava (IVC) puncture, hearts [left ventricle (LV) and left atrium (LA) separately] and lungs were quickly excised and weighed, and then hearts were further dissected in isolating solution (ISO) (1.0 mM MgCl\(_2\), 100.0 mM KCl, 2.0 mM EGTA, 4.0 mM ATP, and 10.0 mM imidazole, pH 7.0; all chemicals from Sigma-Aldrich, St. Louis, MO), snap frozen in liquid nitrogen, and stored at −80°C until further use.

**Measurement of renin, angiotensin-converting enzyme, and angiotensin-converting enzyme 2 activities.** SD (\(n = 6\)) and mRen2 (\(n = 6\)) blood samples were collected by IVC puncture immediately before sacrificing the rats. Blood was gently mixed with citrate buffer immediately after collection. Plasma was separated by centrifugation (15 min, 2,000 \(g\)). Renin activity was measured by a renin assay kit (MAK157, activity kit; Sigma-Aldrich), according to the manufacturer’s instructions. The reaction time was 100 min and fluorescence intensities (excitation: 540 nm; emission: 590 nm) were measured at 120-s intervals. The measured values were plotted as the function of time (min) and fitted by a linear regression. Fit was accepted when \(r^2 > 0.8\). The slope (change in fluorescence intensity in 1 min) was compared with that of recombinant renin. Enzyme activity is expressed in μg/l units, where the μg represents the activity of 1 μg of the recombinant enzyme provided in the kit.

**Data analysis.** Data are presented as means ± SE. Statistical analysis was performed with the one-way ANOVA (Kruskal-Wallis test) followed by the nonparametric Mann-Whitney test. *P < 0.05, statistical differences (unpaired comparison, nonparametric Mann-Whitney test).
### Table 1. General parameters

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>SD (control) (n = 6)</th>
<th>mRen2 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11th wk</td>
<td>13th wk</td>
</tr>
<tr>
<td><strong>Follow-up</strong></td>
<td></td>
<td></td>
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<tr>
<td>Systolic BP, mmHg</td>
<td>120.18 ± 5.24</td>
<td>141.78 ± 4.89</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>84.36 ± 3.46</td>
<td>99.94 ± 6.50</td>
</tr>
<tr>
<td>HR, beat/min</td>
<td>352.76 ± 9.25</td>
<td>349.13 ± 13.19</td>
</tr>
<tr>
<td>LV mass, g</td>
<td>1.20 ± 0.03</td>
<td>1.18 ± 0.05</td>
</tr>
<tr>
<td>Autopsy</td>
<td></td>
<td></td>
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<tr>
<td>LV weight/BW, mg/g</td>
<td>— —</td>
<td>— —</td>
</tr>
<tr>
<td>LA weight/BW, mg/g</td>
<td>— —</td>
<td>— —</td>
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<tr>
<td>Lung weight/BW, g/g</td>
<td>— —</td>
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</table>

Data are given as means ± SE for follow-up (5 repeated measurements from 6 animals) and as mean ± SE for autopsy. SD, male age-matched control Sprague-Dawley rats; mRen2, male homozygous transgenic rats harboring the murine Ren-2 gene (mRen2); BP, blood pressure; HR, heart rate; LV, left ventricular; LA, left atrial; BW, body weight. *P < 0.05, statistically significant differences between the groups (SD vs. mRen2, Mann-Whitney test, nonparametric). †P < 0.05, statistically significant differences during the follow-up period (vs. initial time point) according to ANOVA on ranks (nonparametric Kruskal-Wallis test).

About 0.1 g (wet tissue) of deep frozen LV heart samples (n = 4 from both SD and mRen2 rats) were homogenized (Pro200 homogenizer; ProScientific, Oxford, CT) in 10 vol of ice cold Dulbecco’s phosphate-buffered saline (DPBS, Ca²⁺ and Mg²⁺ free; Gibco, Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined by bicinchoninic acid using bovine serum albumin (BSA; from Sigma-Aldrich) as a standard. Protein concentrations were ~20 mg/ml. The reaction mixture for the measurement of angiotensin-converting enzyme (ACE) activity contained 6 μl heart homogenate in a buffer of 100.0 mM Tris·HCl, 15.0 mM NaCl, and 10.0 μM ZnCl₂ at pH 7.0. The reaction time was 45 min and fluorescence intensities (excitation: 320 nm; emission: 405 nm) were measured at 60-s intervals. The measured values were plotted as the function of time and fitted by a linear regression. ACE activity was calculated by the equation:

\[
ACE\ activity = \left( \frac{S}{k} \right) \times D/P
\]

where S is the rate of increase in fluorescence intensity (slope), k is the increase in fluorescence intensity when 1 μmol of the substrate is

Fig. 3. Echocardiographic assessment of LV systolic function and left atrial (LA) dimensions. Parasternal long axis (PLAX) view was recorded in SD (n = 6) and mRen2 (n = 6) rats (representative examples in A). LV ejection fraction (EF; B) and fractional shortening (FS; C) were determined offline in M-mode according to LV internal diameter upon diastole and systole. Apical 3-chamber (A3C) view in 2-dimensional mode was applied to visualize LA (indicated by dashed blue lines in representative examples in D). LA internal area was calculated based on end-diastolic LA dimensions (E). Symbols are mean of the mean ± SE (n = 3 repeated measurements for each single value). Please note, that although error bars are plotted for each data point, they are overlapped by the symbols (invisible) in some cases and that there were no statistical differences observed neither within (ANOVA on ranks, nonparametric Kruskal-Wallis test) or between (unpaired, nonparametric Mann-Whitney test) the groups.
being cleaved, $D$ is the dilution factor (35 in these experiments), and $P$ is the protein concentration (in mg/ml); 1 unit (U) represents 1 nmol substrate cleavage in 1 min by 1 mg of protein.

ACE2 activity was measured similarly as described earlier (39). In short, 20 μl of tissue homogenate were added to the reaction mixture containing protease inhibitors: 10.0 μM Bestatin-hidrochloride; 10.0 μM Z-protly-prolin (Enzo Life Sciences, Exeter, UK), 5.0 μM Arnastatin-hidrochloride, 10.0 μM Captopril and 5.0 mM NaCl, 100.0 μM ZnCl2, and 75.0 mM Tris-HCl, pH 6.5. All chemicals were from Sigma-Aldrich if not stated otherwise. Reaction temperature was 37°C. Fluorescence intensities (excitation: 320 nm; emission: 405 nm) were measured in 6-min intervals for 84 min. Values were plotted as a function of time (min) and fitted by a linear regression. Fit was accepted when $r^2 > 0.8$. ACE2 activity was calculated according to the equation:

$$\text{ACE2 activity} = \left( \frac{S}{k} \right) \times \frac{D}{P}$$

where $S$ is the rate of increase in fluorescence intensity (slope), $k$ is the increase in fluorescence intensity when 1 nmol of the substrate is being cleaved, $D$ is the dilution factor (10 in these experiments), and $P$ is the protein concentration (in mg/ml); 1 unit (U) represents 1 nmol substrate cleavage in 1 min by 1 mg of protein.

Echocardiography. Cardiac functional tracking was implemented by a General Electric Vivid E9 ultrasound system equipped with a linear 14.1-MHz i13L probe (General Electric, Fairfield, CT). Echocardiographic procedure was performed under light anesthesia by a General Electric Vivid E9 ultrasound system equipped with a linear 14.1-MHz i13L probe (General Electric, Fairfield, CT). Echo-cardiographic procedure was performed under light anesthesia by a single observer blind to subject identity. Parasternal long axis M-mode was obtained at the level of the papillary muscles to asses LV mass (g) and systolic function, such as LV ejection fraction (EF, %) and fractional shortening (FS, %). Trace function in two-dimensional apical three-chamber view was applied to estimate largest LA dimensions, such as end-diastolic LA internal area. Subsequently, color Doppler mode in apical four chamber view was applied to estimate LV diastolic parameters at the level of the mitral valve upon cardiac relaxation. Early diastolic filling peak velocity (E, m/s), late (i.e., atrial) filling peak velocity (A, m/s), deceleration time (DT, ms), and isovolumetric relaxation time (IVRT, ms) were recorded by pulsed-wave Doppler. Calculation by formula $4 \times V^2$ was used to estimate A-peak LA-LV pressure gradient through the mitral valve during late phase LV diastole (35). Early diastolic annular velocity ($a'$, mm/s) and late diastolic annular velocity ($a''$, mm/s) were captured by tissue Doppler at the level of the mitral annulus. Echo images were analyzed and cardiac indexes were calculated offline by EchoPAC clinical workstation software (General Electric).

Histology. Both SD (n = 5) and mRen2 (n = 5) hearts were fixed in formaldehyde (3.6% in phosphate buffer) for 2 days and then the regions of interest (where the coronary arteries come out to the pericardial surface) were cut out. The paraffin-embedded slices were cut to 4-μm slices for further staining. All chemicals for histology were purchased from Sigma-Aldrich. Hematoxylin and eosin staining was done according to Mayer. Jones’ staining (24), Gomori’s staining (15), and elastica van Gieson (EVG)/Weigert’s Resorcin-Fuchsin staining (41) of the slides were performed according to the standard protocols. Jones’ methenamine silver technique demonstrates base-ment membrane and collagen, Gomori’s silver impregnation stain shows reticular fibers, while EVG staining shows elastic fibers within the extracellular matrix.

Force measurements on isolated cardiomyocytes. Isometric force generation of single LV cardiomyocytes was measured at sarcomere lengths of 2.3 μm, according to formerly described method by Papp et al. (36). Briefly, deep frozen (−80°C) LV tissue samples from SD and mRen2 hearts were mechanically disrupted in 0.5% Triton X-100 detergent for 5 min, and subsequently washed in ISO again at 4°C. Myocyte-sized preparations were mounted with silicone adhesive between a high-speed length controller (Aurora Scientific, Aurora, Canada) and a precise force transducer (SensoNor, Horten, Norway) in ISO at 15°C. Cardiomyocyte Ca2+-activated force generation was evoked by triggering the preparation from relaxing (10.0 mM BES, 37.11 mM KCl, 6.41 mM MgCl2, 7.0 mM EGTA, 6.94 mM ATP, and 15.0 mM creatine phosphate, pH 7.2) to activating solution (containing Ca2+-EGTA instead of EGTA, otherwise same composition as relaxing solution) (30). Ca2+ concentrations were expressed as $-\log_{10}[\text{Ca}^{2+}]$ (pCa) units, accordingly the pCa of relaxing solution was 9.0, whereas the pCa of maximal activating solution was 4.75. All solutions were supplemented with protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 40.0 μM leupeptin, and 10.0 μM E-64. All chemicals were purchased from Sigma-Aldrich.

Maximal Ca2+-activated force ($F_{max}$) was induced at pCa 4.75, while submaximal Ca2+-activated force ($F_{active}$) generation was registered at pCa <4.75 by applying activating solutions with lower Ca2+ concentrations. Experiments were recorded and analyzed by custom-built LABVIEW Data Acquisition platform and LabVIEW analyzing software (National Instruments, Austin, TX). $F_{active}$ values

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Table 2. Echocardiographic parameters of SD and mRen2 rats

<table>
<thead>
<tr>
<th>Systolic indexes</th>
<th>SD (control) (n = 6)</th>
<th>mRen2 (n = 6)</th>
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<tbody>
<tr>
<td></td>
<td>11th wk</td>
<td>13th wk</td>
</tr>
<tr>
<td>EF, %</td>
<td>66.40 ± 1.25</td>
<td>69.60 ± 1.91</td>
</tr>
<tr>
<td>FS, %</td>
<td>32.20 ± 1.02</td>
<td>35.00 ± 1.41</td>
</tr>
<tr>
<td></td>
<td>11th wk</td>
<td>13th wk</td>
</tr>
<tr>
<td></td>
<td>68.00 ± 1.48</td>
<td>67.60 ± 1.29</td>
</tr>
<tr>
<td></td>
<td>33.8 ± 1.14</td>
<td>33.40 ± 0.75</td>
</tr>
<tr>
<td>Diastolic indexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA area, mm²</td>
<td>26.31 ± 0.77</td>
<td>28.06 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>1.85 ± 0.11</td>
<td>1.84 ± 0.10</td>
</tr>
<tr>
<td>Pressure gradient, mmHg</td>
<td>0.59 ± 0.06</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>DT, ms</td>
<td>47.10 ± 3.24</td>
<td>46.62 ± 2.56</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>33.60 ± 0.94</td>
<td>31.48 ± 0.77</td>
</tr>
<tr>
<td>e′</td>
<td>1.16 ± 0.09</td>
<td>1.16 ± 0.14</td>
</tr>
<tr>
<td>e′/a′</td>
<td>22.93 ± 2.69</td>
<td>24.85 ± 3.06</td>
</tr>
<tr>
<td>A/a′</td>
<td>12.51 ± 1.06</td>
<td>11.82 ± 0.80</td>
</tr>
</tbody>
</table>

Data are given as means ± SE. EF, LV ejection fraction; FS, LV fractional shortening; E, peak of early LV diastolic filling; A, peak of late diastolic LV filling; DT, deceleration time; IVRT, isovolumetric relaxation time; e′, early diastolic mitral annular velocity; a′, late diastolic velocity at the mitral annulus. Calculated A peak LA-LV pressure gradient during LV diastole is indicated as pressure gradient. *$P < 0.05$, statistical differences between the groups (SD vs. mRen2; Mann-Whitney test, nonparametric). †$P < 0.05$, statistically significant differences during the follow-up period (vs. initial time point) evaluated by ANOVA on ranks (nonparametric Kruskal-Wallis test).
at pCa <4.75 were normalized to $F_{\text{max}}$ (normalized $F_{\text{active}}$) and fitted to a modified Hill equation in Origin 6.0 analysis program (OriginLab, Northampton, MA) providing the Ca$^{2+}$ sensitivity curve for each individual cell. Accordingly, pCa value for the half-maximal Ca$^{2+}$-induced contraction indicated by pCa$_{50}$ defines per se the Ca$^{2+}$ sensitivity of force production of the contractile machinery. Following each Ca$^{2+}$-dependent force development, cardiomyocyte Ca$^{2+}$-independent passive tension ($F_{\text{passive}}$) corresponding to cellular relaxation was measured by the shortening to 80% of initial preparation length for 8 s in relaxing solution. For every single cell, original forces were normalized to myocyte cross-sectional area indicating absolute force values of $F_{\text{max}}$, $F_{\text{active}}$, and $F_{\text{passive}}$ expressed in kN/m$^2$. LV samples from four different hearts ($n = 5–6$ cardiomyocytes from each heart) were used in the force measurements.

**Titin assays.** Titin isoform and phosphorylation analyses were done as previously described (4, 20) with slight modifications. LV tissue samples ($n = 4$ in duplicates) from SD and mRen2 animals were prepared as for the mechanical measurements and dissolved in sodium dodecyl sulfate (SDS) sample buffer [8.0 M urea, 2.0 M thiourea, 3.0% SDS, 75.0 mM dithiothreitol (DTT), 0.05 M Tris·HCl (pH 6.8), 10.0% glycerol, 0.004% brome-phenol blue, 40.0 $\mu$M leupeptin, and 10.0 $\mu$M E-64; all from Sigma-Aldrich]. After centrifugation (16,000 g for 5 min at 24°C), protein amount of supernatant was estimated by dot-blot technique, and set to 2 mg/ml concentration correlating the sample to BSA standards. Polyacrylamide gel electrophoresis (PAGE) was performed by using 2% agarose-strengthened gel to separate titin. Gels were run at 2 mA (constant current) for 540 min. Gels were stained with Coomassie brilliant blue (Reanal, Budapest, Hungary) protein gel stain for titin identification and isoform analysis.

Gels were also stained for 90 min with Pro-Q Diamond (Invitrogen, Molecular Probes, Eugene, OR) phospho-protein gel stain to estimate total titin phosphorylation. Then, gels were washed and handled according to the manufacturer’s protocol. Beyond that, Western immunoblotting was applied to assess titin phosphorylation at particular serine (Ser) residues of PEVK segment (rich in proline, glutamate, valine and lysine amino acids) (30). The phospho-specific antibodies were validated by Granzier et al. (20, 22). Phospho-specific antibodies were developed against Ser-11878 (PS26; GL Biochem, Shanghai, China; 1:1,000) and Ser-1202 (PS170; Genscript, Piscataway, NJ; 1:1,000) in the full human sequence. These human Ser-11878 and Ser-1202 phospho-sites correspond to Ser-12742 and Ser-12884 in the rat genome, respectively (19). Finally, peroxidase-labeled secondary antibody (anti-rabbit-POD; from Sigma-Aldrich; 1:4,000) and enhanced chemiluminescence reaction (ECL) were used for detection. Protein bands were documented by an MF-ChemiBIS 3.2 gel documentation system (DNR Bio-Imaging Systems, Jerusalem, Israel). Signal intensities of titin identified exclusively on 2% gels were evaluated by ImageJ image processing program (National Institutes of Health).

**Fig. 4.** Echocardiographic evaluation of LV diastolic function by pulsed-wave (PW) Doppler. PW Doppler on mitral inflow in apical 4-chamber (A4C) view was used to assess LV relaxation in SD ($n = 6$) and mRen2 ($n = 6$) animals (representative echo images on A and B, respectively). Peak early filling (E) and late diastolic filling (A) velocities were determined at the level of mitral valve upon LV diastole. Deceleration time (DT) represents the descending phase of E wave (oblique dotted white lines), while isovolumetric relaxation time (IVRT) corresponds to the phase following the end of aortic ejection until the mitral opening (horizontal solid white lines). E/A ratio ($C$), calculated A peak LA-LV pressure gradient ($D$), DT ($E$), and IVRT ($F$) are shown. Symbols represent means ± SE ($n = 3$ repeated measurements for each single value). Please note, that although error bars are plotted for each data point, they are overlapped by the symbols (invisible) in some cases. *$P < 0.05$, statistical differences between the mRen2 and SD groups (unpaired comparison, nonparametric Mann-Whitney test). **$P < 0.05$, significant changes in the parameters during the follow-up period (ANOVA on ranks, nonparametric Kruskal-Wallis test).
Health, Bethesda, MD). Optical densities were converted to numerical values as area under the curve by MagicPlot software (Magicplot Systems, Saint Petersburg, Russia). Afterwards, total phosphorylation status of titin was determined by the ratio of signals related to phospho-protein by Pro-Q Diamond and total protein amount by Coomassie blue. Similarly, ECL signals of site-specific phosphorylation of titin’s PEVK element were normalized to Western blot stain referring to the entire titin protein amount transferred. Thus titin phosphorylation was represented in relative terms between SD and mRen2 groups.

**Protein kinase C-α assay.** About 0.1 g (wet tissue) of deep frozen LV heart samples (n = 4 from both SD and mRen2 rats) were homogenized (Pro200 homogenizer; ProScientific, Oxford, CT) in 10-fold diluted ice cold DPBS (Ca²⁺ and Mg²⁺ free; GIBCO, Thermo Fisher Scientific). Homogenized samples were supplemented by equal volume of twofold concentrated SDS sample buffer (Sigma-Aldrich) and incubated for 10 min at 100°C. Fifty micrograms of proteins were loaded onto 10% SDS-polyacrylamide gels. Membranes were blocked by 3% BSA (Sigma-Aldrich) dissolved in Tris-buffered saline (TBS; Sigma-Aldrich). Protein kinase C-α (PKCα) expression was tested by anti-PKCα (Sigma-Aldrich; 1:20,000) (32) and anti-phospho(Ser-657/Tyr-658)-PKCα (EMD Millipore, Temecula, CA; 1:500) antibodies. Actin expression was tested by an anti-actin antibody (Sigma-Aldrich; 1:3,500). Signals were detected and processed as described for titin above.

**Statistical analysis.** All data and statistical differences were calculated and displayed by GraphPad Prism 5.02 software (GraphPad Software, La Jolla, CA). The number of observations was low in the study precluding proper evaluation of normality. Therefore, nonparametric tests were used for the statistical evaluation. Age-matched SD and mRen2 groups were compared by unpaired Mann-Whitney nonparametric test, while group follow-up data were addressed by ANOVA on ranks (Kruskal-Wallis, nonparametric test). Statistical significance was accepted and shown when P < 0.05. Bars and symbols on the graphs represent the mean ± SE.

**RESULTS**

**Higher activity of the RAAS in transgenic rats harboring the mRen2.** The activity of some of the key elements of the RAAS was measured. Plasma renin activity was elevated in the mRen2 rats compared with age-matched SD control rats (17.64 ± 2.12 μg/l equivalent in mRen2 vs. 12.66 ± 2.64 μg/L equivalent in SD, P < 0.05; Fig. 1A). LV ACE activity (responsible for cardiac ANG I to ANG II conversion) was similar in both mRen2 and SD rats (0.261 ± 0.022 U/mg in mRen2 vs. 0.216 ± 0.024 U/mg in SD, P = 0.20; Fig. 1B). In contrast, LV ACE2 activity (responsible for the elimination of ANG II in the heart) was lower in mRen2 rats compared with SD rats (2.49 ± 0.40 vs. 3.82 ± 0.31 U/mg in SD, P < 0.05; Fig. 1C).

**Pronounced HTN in mRen2 animals.** Both systolic and diastolic BP was severely high from the 11th wk of age in the mRen2 group compared with the SD group (Fig. 2, A and B; Table 1). This increase in BP was paralleled by an increase in HR in mRen2 rats (Fig. 2C; Table 1). Interestingly, some of these parameters were further increased during the 4-wk follow-up period in the mRen2 rats (Fig. 2, B and C).

**Impaired cardiac relaxation with preserved systolic function in mRen2 rats in vivo.** Transgenic mRen2 rats had similar EF and FS than SD rats (at 15th wk P = 0.53 and P = 0.61, respectively; Fig. 3, B and C; Table 2). There was no difference in LA area in mRen2 and SD rats (at 15th wk P = 0.24; Fig. 3D).

![Fig. 5. Echocardiographic evaluation of LV diastolic function by mitral annulus tissue Doppler (TD). Mitral annulus TD in A4C view was applied to assess LV relaxation in SD (n = 6) and mRen2 (n = 6) animals (representative echo images on A and B, respectively). Early diastolic annular velocity (e’) and late diastolic velocity (a’) were measured at the mitral annulus upon LV diastole. Ratios of e’/a’ (C), E/e’ (D), and A/a’ (E) were calculated. Symbols represent mean of the mean ± SE (n = 3 repeated measurements for each single value). Please note, that although error bars are plotted for each data point, they are overlapped by the symbols (invisible) in some cases. *P < 0.05, statistical differences between the mRen2 and SD groups (unpaired comparison, nonparametric Mann-Whitney test). No significant changes were observed in the parameters during the follow-up period (ANOVA on ranks, nonparametric Kruskal-Wallis test).](http://ajpheart.physiology.org/)

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3E and Table 2). In contrast, a deteriorated diastolic function was found in mRen2 subjects. Ratio of early and late LV diastolic filling velocities (E/A) of mRen2 hearts was lower (Fig. 4C and Table 2), primarily determined by a prominent increase in atrial filling velocities. Accordingly, there was a significant difference in the calculated A-peak LA-LV pressure gradient in these rats (Fig. 4D and Table 2). DT was higher (Fig. 4E and Table 2) and IVRT was longer (Fig. 4F and Table 2) than that of those in the age-matched SD group. Interestingly, other diastolic parameters, such as the ratio of early and late diastolic mitral annular velocities (e'/a') (at 15th wk $P = 0.75$; Fig. 5C and Table 2) and the E/e' as indicative for LV diastolic filling pressure (at 15th wk $P = 0.42$; Fig. 5D and Table 2) values, were similar. In contrast, compared with SD, the A/a' ratio of mRen2 hearts became significantly increased during the follow-up period (Fig. 5E and Table 2).

**Cardiopulmonary morphological adaptation in mRen2 rats.** LV mass was estimated by echocardiography and showed a gradual increase in mRen2 rats until the 15th wk, becoming significantly higher than that at the 11th wk and that of in age-matched SD controls (Fig. 6A and Table 1). This was in accordance with the LV weight (mg)/BW (g) ratios at the 15th wk (Fig. 6B and Table 1). This represented LV hypertrophy, since there was no difference in the BW among these groups (363.86 ± 18.29 of mRen2 and 379.86 ± 15.44 g of SD rats, $P = 0.52$). Furthermore, LA weight (mg)/BW (g) and lung weight (mg)/BW (g) ratios of mRen2 rats were significantly increased as well (Fig. 6, C and D; Table 1).

**Hypertrophy and perivascular fibrosis in the LV of mRen2 rats.** LV tissue sections were stained by hematoxylin and eosin. Cardiomyocyte hypertrophy was observed without other large-scale deterioration of myocardial structure in mRen2 rats (Fig. 7A). Jones' staining of the basement membranes revealed collagen deposition in the perivascular regions (Fig. 7B). Gomori’s stain of the reticular fibers supported the observed perivascular accumulation of extracellular matrix components (Fig. 7C). Finally, EVG staining was used to visualize elastic fibers (Fig. 7D). Again, accumulation of these fibers was apparent in the perivascular regions.

**Unaffected $Ca^{2+}$-activated force generation in single cardiomyocytes from mRen2 hearts in vitro.** $Ca^{2+}$-activated force generation was studied in skinned cardiomyocytes isolated from LV tissue samples in vitro. $Ca^{2+}$-activated maximal force generation ($F_{\text{max}}$) was similar in mRen2 and age-matched SD rats (25.886 ± 0.989 and 29.054 ± 2.798 kN/m², respectively, $P = 0.33$; Fig. 8, A and B). $Ca^{2+}$ sensitivity of force production was also similar (5.825 ± 0.013 in mRen2 and 5.873 ± 0.053 in SD rats, $P = 0.42$; Fig. 8, C and D).

**Increased $Ca^{2+}$-independent (passive) tension in mRen2 hearts in vitro.** In contrast to the unaffected $Ca^{2+}$-activated force generation, $Ca^{2+}$-independent tension ($F_{\text{passive}}$) was increased in the LV of mRen2 rats (1.741 ± 0.063 kN/m²), compared with age-matched SD rats (1.276 ± 0.176 kN/m², $P < 0.05$; Fig. 9A).

**Selective hyperphosphorylation of Ser-12742 within the PEVK element of titin in mRen2 hearts in vitro.** Protein gel staining (Fig. 9C) or blot staining (Fig. 9, D and E) showed similar titin expression pattern in mRen2 and SD hearts. The major band representing the N2B isoform of titin was detected at ~3.0 MDa of molecular weight, which was accompanied by a much weaker band (titin T2) in some cases. Subsequent phospho-protein sensitive staining with Pro-Q Diamond showed similar levels of phosphorylation in mRen2 hearts when normalized to the signal in SD hearts on the same gels (0.920 ± 0.037 vs. 1.000 ± 0.038, respectively, $P = 0.14$; Fig. 9C). Similarly, no difference was found in the normalized phosphorylation level at Ser-12884 (0.922 ± 0.140 vs. 1.000 ± 0.083 in SD, $P = 0.63$; Fig. 9E) within the PEVK element of titin. In contrast, mRen2 myocardium exhibited a higher phosphorylation level at Ser-12742 (1.326 ± 0.117 vs. 1.000 ± 0.063 in SD, $P < 0.05$; Fig. 9D) in the same (PEVK) region.

**Increased PKCa expression in mRen2 hearts in vitro.** PKCa expression and phosphorylation was tested to provide a potential mechanism for increased titin phosphorylation. PKCa expression was increased by 1.783 ± 0.135-fold in mRen2 rats compared with SD (1.000 ± 0.122, $P < 0.05$; Fig. 10A). No difference in the level of phosphorylated (Ser-657/Tyr-658) PKCa was found when normalized to actin expression (1.252 ± 0.165 in mRen2 vs. 1.000 ± 0.062 in SD, $P = 0.77$; Fig. 10B) or when normalized to PKCa expression (0.794 ± 0.063 in mRen2 vs. 1.000 ± 0.098 in SD, $P = 0.16$; Fig. 10C).

**DISCUSSION**

It was confirmed here that mRen2 rats have severely high systolic and diastolic BP compared with the age-matched control group (SD), in accordance with the first report on this
animal model (34). Our data support the idea that transgenic mRen2 rats have an increased plasma renin activity, which may directly activate the circulating RAAS. Moreover, we found a decrease in cardiac ACE2 activities responsible for ANG II breakdown, besides to similar ACE activities in the mRen2 rats. Therefore, in line with former results (47), these findings suggest that cardiac elimination of ANG II is lower in mRen2 animals, potentially increasing local RAAS activity.

LV hypertrophy is another prominent feature of mRen2 rats (34), which was confirmed in our experiments. In this respect, there are studies suggesting functional expression of the transgene in the myocardium, providing a direct link between the genotype and phenotype (9, 28), although one may argue that severe HTN (cardiac overload) can lead to cardiac hypertrophy independently of the myocardial RAAS.

In spite of the substantial efforts, there are controversial reports on the cardiac performance in the mRen2 strain. Earlier reports using cine magnetic resonance imaging (MRI) and echocardiography described reduced LV EF in these transgenic rats (12, 43). Some potential molecular mechanisms were proposed for this phenotype, including sustained ANG II-mediated oxidative stress (43), downregulation of β-adrenergic receptors associated with reduced positive inotropic response of papillary muscles to isoproterenol (7, 46), and decreased Ca²⁺ sensitivity of skinned fibers (8, 46). In contrast, some recent reports suggested no change in EF in the mRen2 strain according to cine MRI investigations (16, 31) or in LV FS in ovariectomized female mRen2 rats by echocardiography (23). Our data are in accordance with these latter articles, as we found no difference in EF or FS in mRen2 and age-matched SD rats.

The same reports, which found no effects on systolic function, showed altered diastolic function in mRen2 rats (16, 23, 31). This feature was also prominent in our studies. In partic-
Fig. 8. Ca$^{2+}$-activated force production of isolated LV single cardiomyocytes. Isometric Ca$^{2+}$-activated force generation ($F_{\text{active}}$) at sarcomere lengths of 2.3 μm is plotted as a function of the applied $-\log\text{[Ca}^{2+}]$, i.e., pCa (A). Maximal Ca$^{2+}$-activated force ($F_{\text{max}}$) (force at the highest applied pCa = 4.75) is shown on the bar graph in B. $F_{\text{active}}$ was normalized to the $F_{\text{max}}$ in each cell to visualize the Ca$^{2+}$ sensitivity of force production (C). Half-maximal values of the Ca$^{2+}$ sensitivity of force production (pC$\text{a}_{50}$) are shown in D. Values represent mean of the mean ± SE (n = 4 hearts from each group; n = 5–6 cardiomyocytes from each heart). Note, that there were no statistical differences (unpaired, nonparametric Mann-Whitney test) observed.

...ular, we found a significantly decreased LV early and late diastolic filling (E/A) ratio, increased DT, and prolonged IVRT. Moreover, a slight, but significant, increase in lung weight (as normalized to the BW) suggests pulmonary congestion. As a matter of fact, these echocardiographic data are characteristic for human HF with preserved EF, i.e., HFpEF (5–6, 37, 44). On the other hand, we did not find any differences in diastolic parameters directly related to the ventricular wall motion and characteristic for human HFpEF, such as E/e' or e'/a'. This obvious controversy might be explained by different adaptation to prolonged HTN. Prolonged HTN in human often results in pressure overload in the LA, which is dilating upon time, resulting in lower LV filling pressures, lower A-wave amplitudes, and pseudonormalization of E/A values. This is not the case in the mRen2 rats. These rats apparently adapt to the increased ventricular stiffness by thickening of the atrial wall (LA hypertrophy) and by increasing late LV filling velocity (increased A wave) and pressure gradient. Hence, our observations in this experimental model might contribute to greater understanding of the mechanism of preclinical diastolic dysfunction, a great importance in human (42).

We have investigated the molecular mechanisms leading to diastolic dysfunction in the mRen2 animal model. The impaired in vivo cardiac relaxation was accompanied by increased LV cardiomyocyte Ca$^{2+}$-independent (passive) tension (a measure of cellular diastolic function, $F_{\text{passive}}$), without significant changes in the Ca$^{2+}$-activated force production (a cellular measure of systolic function). Indeed, reports on small cardiac biopsies from HF patients demonstrated that cardiomyocyte $F_{\text{passive}}$ is increased in HFpEF (3) and that $F_{\text{passive}}$ is being higher compared with HF with reduced EF, i.e., HFrEF (40). Accordingly, high cardiomyocyte resting tension is considered as the cellular trademark of diastolic dysfunction seen in HFpEF (5–6, 17–19, 30, 45).

Increased diastolic tension of skinned LV fibers from mRen2 hearts was reported earlier (46). Important to note, stiffness may be related to intracellular and extracellular components in multicellular preparations such as the skinned papillary muscle. In line with the potential contribution of extracellular factors, previous studies suggested extracellular collagen accumulation and perivascular fibrosis in mRen2 hearts (2, 14). Indeed, here we also found similar deposition of collagen in the mRen2 hearts. Nonetheless, our mechanical experiments were performed on isolated skinned cardiomyocytes addressing intracellular factors selectively and independently of extracellular matrix for the first time.

The main intracellular determinant of passive tension of the contractile machinery is the titin molecule, spanning the half sarcomere (30). This protein has two isoforms in adult mammalian heart, a larger and more compliant N2BA isoform (~3.3 MDa) and a smaller and stiffer N2B isoform (~3.0 MDa). The predominant titin isoform in adult rats is the N2B (11). Stiffness is modulated by titin isoform composition, degradation, oxidative modifications, and phosphorylation by various protein kinases (30) especially within the elastic I-band region at N2-Bus and PEVK domains (1, 17–22, 26, 45). Titin was directly studied in mRen2 rats here for the first time. Only the N2B titin isoform was expressed without apparent signs of proteolytic degradation; therefore, selective expression of titin isoforms or proteolysis is an unlikely mechanism for the observed increase in cardiomyocyte stiffness. In this context, considering high LV ANG II levels in mRen2 rats (47) it is important to note, that ANG II promotes the transition of titin to the N2B isoform through the phosphatidylinositol-3-kinase-AKT-mammalian target of rapamycin (PI3K-AKT-mTOR) pathway (27). It is therefore possible to say that titin isoform expression is proceeded to produce exclusively the stiff N2B isoform in mRen2 transgenic rats presenting ANG II-dependent HTN.

The N2-Bus domain (a unique spring segment of titin) has various phosphorylation sites for protein kinase A (PKA), protein kinase G (PKG), extracellular signal-regulated kinase-2 (ERK2), and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKIIδ) (30). Hypophosphorylation of this region was observed in HFpEF, leading to increased titin-based stiffness (4, 18–19, 45). However, we found no change in this region as addressed by phospho-specific (Pro-Q Diamond) gel staining in mRen2 rats. ANG II activates PKCα-mediated and CaMKIIδ-driven myofilamental phosphorylation, targeting titin in addition to other myofibrillar proteins (30). PKCα phosphorylates titin’s PEVK spring element at Ser-12742 and Ser-12884, apparently giving rise in cardiomyocyte titin-based stiffness (20). At the same time, PKCα-mediated PEVK phosphorylation was reportedly hinted to have preference at Ser-12742, although both residues seem to have importance in maintaining structural integrity (1). Interestingly, the PEVK region can also be
Fig. 9. Titin-related mechanical and biochemical parameters. Ca\(^{2+}\)-independent diastolic tension in myocyte-sized preparations (\(F_{\text{passive}}\)) was determined at sarcomere lengths of 2.3 μm (A). Bars are mean of the mean ± SE (\(n = 4\) hearts in each group; \(n = 5–6\) cardiomyocytes from each heart). Schematic structure of titin N2B isoform (predominant in rats) is presented on panel B: elastic titin spring is mainly constructed by immunoglobulin-like (Ig) domains and two disordered segments, namely N2B-unique sequence (N2-Bus) and PEVK (proline, glutamate, valine, and lysine reach motif). Two phosphosites (P) within the PEVK domain identified as targets of known protein kinases are indicated according to either the human or rat sequence. Titin isoform expression and total phosphorylation were assessed by Coomassie blue gel staining (total protein) and Pro-Q Diamond phospho-protein sensitive gel staining. The ratio of the optical densities of the bands (phospho-protein/total protein) was normalized to the average of the control (age-matched SD) on each gel. Representative pictures and summarized data (bar graph) are shown in C. Site-specific phosphorylation at Ser-12742 (specific antibody labeling on Western immunoblots (WB) and blot staining. \(N_2B\)-unique sequence (N2-Bus) and PEVK (proline, glutamate, valine, and lysine reach motif). Two phosphosites (P) within the PEVK domain identified as targets of known protein kinases are indicated according to either the human or rat sequence. Titin isoform expression and total phosphorylation were assessed by Coomassie blue gel staining (total protein) and Pro-Q Diamond phospho-protein sensitive gel staining. The ratio of the optical densities of the bands (phospho-protein/total protein) was normalized to the average of values in SD bands. Relative phosphorylation level was addressed by the ratio of phospho(Ser-657/Tyr-658)-PKC \(\alpha\) (rel.) to actin (rel.) within titin’s PEVK element was assessed by the ratio of phosphorylated protein and total protein according to specific antibody labeling on Western immunoblots (WB) and blot staining. Insets: representative pictures taken from the same membrane (line shows discontinuity within the membrane). Ser-12742-P and Ser-12884-P represent phosphorylated serine residues. Titin T2 is the minor degradation fragment of titin seen in some cases. Samples were loaded in duplicates (\(n = 4\) rats from each group), and experiments were repeated at least 3 times. Bars represent the mean ± SE in relative terms (rel.). *\(P < 0.05\), significant differences between the groups (mRen2 vs. SD; unpaired comparison, nonparametric Mann-Whitney test.

Fig. 10. Investigation of protein kinase C-\(\alpha\) (PKC\(\alpha\)) expression and phosphorylation by WB. PKC\(\alpha\) expression or level of phosphorylation was tested in LV heart samples (\(n = 4\) for both SD and mRen2) by anti-PKC\(\alpha\) (A and C), anti-phospho(Ser-657/Tyr-658)-PKC\(\alpha\) (B and C), and anti-actin (A and B) antibodies. Insets: representative images taken from the same membrane, and line shows discontinuity within the membrane (A and B). Band intensities were evaluated to yield information on the relative amount of PKC\(\alpha\) (A) and phospho(Ser-657/Tyr-658)-PKC\(\alpha\) (B) normalized to actin on the same membranes. Note, values were further normalized for the average of values in SD bands. Relative phosphorylation level was addressed by the ratio of phospho(Ser-657/Tyr-658)-PKC\(\alpha\) and PKC\(\alpha\) signals on separate membranes (C). Four samples were evaluated for each group in duplicates (A) or in triplicates (B and C). Bars represent the mean and error bars refer to SE. *\(P < 0.05\), statistical difference (unpaired comparison, nonparametric Mann-Whitney test.

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phosphorylated by CaMKIIδ (21), proposed to be a contributor to diastolic stiffness of the failing myocardium (17).

Previous reports suggested that the PEVK domain cannot be detected by Pro-Q Diamond stain (22). Accordingly, the PEVK phosphorylation was tested here with phospho-specific antibodies against phosphorylated Ser-12742 and Ser-12884 residues. We found increased phosphorylation at Ser-12742, without changes in the phosphorylation level of Ser-12884 in mRen2 hearts. PEVK Ser-12742 hyperphosphorylation was previously demonstrated in mice with transverse aortic constriction (22), in an old HTN dog model (18), as well as in human HF (17, 26). Moreover, a recent translational work implicated the hyperphosphorylation of the very same site (Ser-12742) in human HFpEF (45). It suggested that a significant rise in collagen- and titin-dependent stiffness is paralleled by explicit changes in titin phosphorylation levels, e.g., hypophosphorylation of PKA/PKG sites in the N2-Bus element and PKCα-dependent hyperphosphorylation at Ser-11878 (Ser-12742 in the rat) in the PEVK element.

Here we reported alterations in the phosphorylation of a single amino acid residue in the PEVK element of titin with no changes in titin isoform composition. It is interesting how this single modification may have such an impact on cardiomyocyte stiffness and diastolic function. Regarding to the magnitude of changes, previously published studies found similar differences as reported here. Increase in PEVK element phosphorylation (Ser-12742 in the rat sequence) was 31% in human HTN (45) and 23% in patients with dilated cardiomyopathy (26), being similar to the 32.6% in mRen2 rats as reported here. A more prominent change (~70%) decrease in PEVK phosphorylation level was found in a different site within the PEVK region of titin (Ser-12884) in a metabolic model of HFpEF (19).

Moreover, hypophosphorylation of N2-Bus residues was also noted previously, accompanying PEVK hyperphosphorylation (45). Taken together, it is not clear what site and what level of phosphorylation of titin translates to diastolic dysfunction so far. It appears that titin phosphorylation is determined by a complicated interplay of kinases and phosphatases, which cannot be easily modeled by in vitro treatments.

In addition, the expression level of PKCα was 1.8-fold increased in mRen2 rats without change in the ratio of phosphorylated (Ser-657/Tyr-658) and total PKCα. These suggest higher levels of activated (phosphorylated) PKCα in mRen2 rats, supporting a role for PKCα in the observed higher phosphorylation levels at titin Ser-12742.

In the present study, genetic stimulation of RAAS signaling resulted in HTN selectively associated with LV diastolic dysfunction, characteristic for human HFpEF. Our work suggests that hyperphosphorylation of Ser-12742 in the PEVK element of titin is a common feature of RAAS-mediated experimental HTN with isolated diastolic dysfunction (as reported here) and clinical HFpEF as reported by others.

There are certain limitations of this study. The most important ones are 1) the low number of animals included in most of the determinations (n = 4 – 6); 2) no direct measurement of angiotensin peptides or aldosterone, making it impossible to directly address RAAS activity; 3) there is no direct evidence for HF in these rats: there is only a slight increase in lung weight/BW ratio, and there were no other obvious signs of HF; 4) the apparently different atrial adaptation of the mRen2 animals to pressure overload warrants further, more detailed, studies; 5) it is almost impossible to estimate the contribution of relatively small differences in cellular diastolic tension (such as found here) to the cardiac performance; and 6) there are no accepted echocardiographic guidelines describing standard values for rats, thereby making it hard to diagnose diastolic dysfunction and even more so HFpEF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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