Rapid communication

Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice

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Received 27 February 2007; received in revised form 3 April 2007; accepted 17 April 2007
Available online 21 April 2007
Time for primary review 14 days

Abstract

Objectives: The peptidase action of angiotensin converting enzyme 2 (ACE2) allows it to function as a negative regulator of the renin–angiotensin system. Current pharmacotherapies for human heart failure, such as ACE inhibitors and angiotensin and aldosterone receptor blockers, increase the activity of ACE2 in the heart. In this study, we investigate the mechanism for the age-dependent cardiomyopathy in ACE2 null mice.

Methods and results: Ace2−/− mutant mice develop a progressive age-dependent dilated cardiomyopathy with increased oxidative stress, neutrophilic infiltration, inflammatory cytokine and collagenase levels, mitogen-activated protein kinase (MAPK) activation and pathological hypertrophy. The angiotensin II receptor-1 (AT1) blocker, irbesartan, prevented the dilated cardiomyopathy in aged Ace2−/− mutant mice, confirming a critical role of angiotensin II (Ang II)-mediated stimulation of AT1 receptors. Ang II activation of AT1 receptors triggers G-protein-coupled receptor (GPCR)-activated phosphoinositide 3-kinase gamma (PI3Kγ) and its downstream pathways. We showed that p110γ, the catalytic subunit of PI3Kγ, is a key mediator of NADPH oxidase activation in response to Ang II. The double mutant mice (Ace2−/−/p110γ−/−) exhibited marked reductions in oxidative stress, neutrophilic infiltration, and pathological hypertrophy resulting in myocardial protection, suggesting that PI3Kγ plays a critical role in Ang II-mediated cardiomyopathy.

Conclusions: Our findings demonstrate that the age-dependent cardiomyopathy in ACE2 null mice is related to increased Ang II-mediated oxidative stress and neutrophilic infiltration via AT1 receptors. Our combination of genetic and pharmacological approaches defines a critical role of ACE2 in the suppression of Ang II-mediated heart failure.

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Keywords: Angiotensin converting enzyme 2; Renin–angiotensin system; Angiotensin II; Oxidative stress; Neutrophils; P13 kinase gamma

1. Introduction

The renin–angiotensin system plays a fundamental role in a variety of cardiovascular diseases including hypertension, heart failure and coronary artery disease [1,2]. In human heart failure, activation of the renin–angiotensin system (RAS) has been linked to the initiation and progression of heart disease.
Indeed, angiotensin II (Ang II), acting via AT1 and AT2 receptors, modulates production of reactive oxygen species, impaired myocardial contractility and extracellular matrix remodeling thereby negatively impacting on heart function [3]. The key peptidase action of angiotensin converting enzyme 2 (ACE2) is degradation of Ang I and Ang II to Ang 1–7 and Ang 1–9, respectively, hence functioning effectively as a negative regulator of the RAS [2]. We have previously shown that loss of ACE2 leads to an age-dependent progressive ventricular dilation and reduced systolic performance [2,4]. ACE2 also plays a critical role in acute lung injury [5] and in the development of glomerosclerosis and kidney disease [6] by promoting Ang II-mediated injury.

In human heart failure, expression of ACE2 is upregulated which plays an important role in counterbalancing an activated RAS [7–9]. Several key pharmacotherapeutic agents with proven efficacy in the treatment of human heart failure including ACE inhibitors, angiotensin receptor and aldosterone receptor blockers enhance ACE2 activity and/or expression [10,11]. Moreover, polymorphisms in the ACE2 gene have also been linked to the development of pathological myocardial hypertrophy and heart disease in humans [12,13]. Here we show that loss of ACE2 leads to a progressive Ang II-mediated dilated cardiomyopathy associated with increased oxidative stress, neutrophilic infiltration, MAPK activation and pathological hypertrophy at 6 and 12 months of age. Long-term blockade of the AT1 receptors prevents the molecular and functional deterioration in ACE2-deficient mice proving that these receptors are the key mediators of the detrimental effects of Ang II. Furthermore, inactivation of the GPCR-coupled isoform, PI3Kγ, leads to marked protection from the detrimental effects of Ang II in the ACE2-deficient heart. These results collectively demonstrate that loss of ACE2 enhances the susceptibility to heart failure due to potentiation of Ang II-mediated injury via AT1 receptors and PI3Kγ. As such, enhancing ACE2 action may serve as a novel therapy for patients with heart failure.

2. Materials and method

2.1. Experimental animals and protocols

Mutant mice have been previously described [4,14] and only male ACE2 mutant (Ace2−/−) and p110γ−/− mutant mice and their littermate wild-type (Ace2+/+) mice were used. Ace2−/− and p110γ−/− mutant mice have a mixed background (Ola129/C57BL/6) and only littermate wild-type controls were used. Ace2−/− mutant and littermate wild-type Ace2+/+ mice were treated with irbesartan in their drinking water starting at 8 weeks of age in order to deliver 50 mg/kg/day over a 10-month period. Irbesartan was kindly provided by Dr R. Santos, Bristol-Myers Squibb, Princeton, NJ. The original Ace2−/− mixed strain was also backcrossed into a pure C57BL/6 background greater than 8 times in order to assess the impact of genetic background. All experiments confirmed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), Institutional Guidelines and the Canadian Council on Animal Care.

2.2. Langendorff perfusion experiments

Ace2−/− mutant and littermate control mice at 6 months of age were anaesthetized with ketamine/xylazine and isolated hearts were immediately placed in cold PBS. Hearts were then perfused in the retrograde Langendorff mode with modified Kreb’s solution (11 mM Glucose, 2 mM Na-Pyruvate, free [Ca2+] = 1.5 mM) in the presence of 95% O2 and 5% CO2 to achieve a final pH of 7.4. A pressure transducer balloon was inserted through the left atrium into the left ventricle and the consequent pressure waveform was recorded at 2 kHz using a Biopac Acquisition system. The balloon was inflated from diastolic of 10 to 40 μL with increments of 5 μL and a stable recording was obtained for 3 min. The temperature was maintained at 36 ± 0.5 °C during all recordings. In order to allow the assessment of NADPH activity, a side arm was used for the infusion of Ang II (30 nM) in p110γ knockout (p110γ−/−) and wild-type littermate (p110γ+/+) mice at 10 weeks of age. Hearts were removed following 5 min of exposure to Ang II and the left ventricles were dissected and snap frozen.

2.3. Echocardiographic and hemodynamic measurements

For the echocardiographic and hemodynamic measurements, mice were lightly anaesthetized with isoflurane/oxygen (1%/99%) and measurements were made as previously described [4]. To examine the inotropic and chronotropic reserve, intraperitoneal bolus injection of the β-receptor agonist dobutamine (1.5 μg/g body weight) was administered and serial M-mode and Doppler images were obtained at 5 min postinjection.

2.4. Measurement of aldehydes

For the measurement of tissue aldehydes, heart samples (100 mg) were ground into a powder in liquid nitrogen and suspended in 0.5 mL of deionized filtered water with EGTA (400 μM; a Ca2+ and iron chelator), butylated hydroxytoluene (20 μM; a lipid peroxidation blocker) and deferoxoxamine (20 μM; an iron chelator). Samples were analyzed by capillary-column gas chromatography–negative ion chemical ionization mass spectrometry as previously described [15].

2.5. Measurement of superoxide production

The chemiluminescence lucigenin assay was used to measure NADPH oxidase activity using a single-tube luminometer (Berthold FB12, Berthold Technologies, Germany) modified to maintain the sample temperature at 37 °C.
as previously described [16,17]. Lucigenin (5 μM) and NADPH (100 μM) were added to the samples and light emission was recorded every 2 min over an 8-minute period. The superoxide scavengers, polyethylene glycol–superoxide dismutase (PEG–SOD; 650 U/mL) and tiron (10 mmol/L), were used to confirm superoxide generation. All measurements were performed in triplicates and results were normalized per 1 mg protein.

2.6. Histology and immunohistochemistry

Trichrome and Picro-Sirius Red (PSR) staining and visualization were carried out as previously described [15,18]. Nitrotyrosine staining was carried out using an antirat nitrotyrosine antibody (1:200; Upstate Biotechnology Inc., Lake Placid, New York) followed by secondary staining with biotinylated antirabbit IgG followed by streptavidin–horseradish peroxidase. The extent of positive nitrotyrosine staining shown as a percentage of the total histological field was quantified using computerized planimetry and color–subtractive image analysis as previously described [15]. Neutrophil staining was carried out using a rat antineutrophil antibody (Serotec, Raleigh, NC) and macrophage staining using Mac3 and F4/80 antibodies as previously described [18]. Myocardial tissue was also stained using rat antimonoclonal antibody (Pharmingen) against platelet-endothelial cell adhesion molecule (PECAM-1). Capillary counts were obtained from 3 sections from each heart and compared to the number of nuclei in the same field.

2.7. Taqman real time PCR and western blot analysis

RNA levels of the indicated genes were determined as previously described [18]. 18S rRNA was used as the internal standard. Western blot analysis of MAPK was carried out as previously described [19] using commercial antibodies from Cell Signaling Inc.

2.8. Statistical analysis

All data are shown as mean±SEM. All statistical analyses were performed using SPSS software (Chicago, Illinois; Version 10.1). The effects of genotype and irbesartan were evaluated using ANOVA followed by the Student Neuman–Keuls test for multiple comparison testing and comparison between two groups was made using the Student’s t test.

3. Results

3.1. Progressive heart failure in aged Ace2−/− mutant mice is Ang II-dependent

Loss of ACE2 leads to an age-dependent and progressive decline in cardiac function associated with ventricular dilation at 6 months and 12 months of age as detected by echocardiographic and hemodynamic measurements (Table 1). Impaired left ventricular function in aged Ace2−/− mutant mice (Fig. 1a) was further confirmed by ex-vivo Langendorff measurements showing a rightward displacement of the pressure–volume curve (Fig. 1b) indicating reduced systolic function (Fig. 1c and d). In agreement with our previous in-vivo findings [4], these results confirm that lack of ACE2 leads top progressive left ventricular dilation and reduced systolic performance with increasing age. The development of cardiomyopathy was associated with eccentric pathological hypertrophy as shown by the increased LV weight normalized to tibal length (Fig. 1e) or body weight (Table 1) with no difference in cardiomyocyte cross-sectional area.

### Table 1

<table>
<thead>
<tr>
<th>Aged mice (C57BL/6/OlaL29 background)</th>
<th>Ace2+/y</th>
<th>Ace2−/−</th>
<th>Ace2−/−+Irb</th>
<th>Ace2+/y</th>
<th>Ace2−/−</th>
<th>Ace2−/−+Irb</th>
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<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
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</tr>
<tr>
<td>Age</td>
<td>6 months</td>
<td>6 months</td>
<td>6 months</td>
<td>12 months</td>
<td>12 months</td>
<td>12 months</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>553±11.4</td>
<td>567±10.6</td>
<td>549±9.2</td>
<td>554±10</td>
<td>559±12.4</td>
<td>569±9.3</td>
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<tr>
<td>PWT (mm)</td>
<td>0.73±0.01</td>
<td>0.71±0.02</td>
<td>0.74±0.02</td>
<td>0.76±0.02</td>
<td>0.75±0.04</td>
<td>0.74±0.03</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.92±0.06</td>
<td>4.67±0.08*</td>
<td>4.01±0.07</td>
<td>3.98±0.06</td>
<td>5.32±0.12*</td>
<td>4.35±0.09</td>
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<tr>
<td>LVESD (mm)</td>
<td>1.82±0.05</td>
<td>3.03±0.05*</td>
<td>1.84±0.07</td>
<td>1.93±0.05</td>
<td>3.13±0.07*</td>
<td>2.2±0.06</td>
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<tr>
<td>FS (%)</td>
<td>54.1±1.8</td>
<td>37.4±2.3*</td>
<td>50.6±2.1</td>
<td>55.7±1.4</td>
<td>31.8±2.5*</td>
<td>48.7±2.4</td>
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<tr>
<td>PAVc (circ/s)</td>
<td>10.8±0.3</td>
<td>7.2±0.31*</td>
<td>10.4±0.42</td>
<td>10.2±0.3</td>
<td>6.61±0.27*</td>
<td>9.2±0.31</td>
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<tr>
<td>PAVc (cm/s)</td>
<td>98.8±1.4</td>
<td>79.8±1.5*</td>
<td>94.3±2.1</td>
<td>104.1±1.8</td>
<td>70.5±2.5*</td>
<td>93.9±3.1</td>
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<td>LVESD/mg (mg Hg)</td>
<td>4.33±0.59</td>
<td>8.17±0.68*</td>
<td>3.29±0.67</td>
<td>4.09±0.72</td>
<td>9.02±0.81*</td>
<td>4.59±0.74</td>
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<tr>
<td>+dP/dt/−dP/dt (mm Hg/s)</td>
<td>10,676±305</td>
<td>7783±317*</td>
<td>10,664±475</td>
<td>11,129±309</td>
<td>6152±523*</td>
<td>9842±619</td>
</tr>
<tr>
<td>−dP/dt (mm Hg/s)</td>
<td>10,891±268</td>
<td>7488±242*</td>
<td>10,026±401</td>
<td>10,687±315</td>
<td>5998±454*</td>
<td>9405±551</td>
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<td>LVW/LW (mg/g)</td>
<td>3.11±0.08</td>
<td>4.07±0.08*</td>
<td>3.43±0.1</td>
<td>3.03±0.11</td>
<td>4.83±0.19*</td>
<td>3.38±0.12</td>
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<tr>
<td>LVW/L (mg/mm)</td>
<td>4.79±0.71</td>
<td>6.31±0.2*</td>
<td>4.81±0.15</td>
<td>4.71±0.1</td>
<td>7.21±0.26*</td>
<td>4.84±0.82</td>
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</tbody>
</table>

Irb = Irbesartan; N = number of mice per group; HR = Heart Rate; PWT = Posterior Wall Thickness; LVEDD, LVESD = Left ventricular end diastolic and systolic dimension, respectively; FS = Fractional shortening = (LVEDD – LVESD)/LVEDD × 100%; VCFc = Velocity of circumferential shortening corrected for HR = FS/ETc; PAVc = Peak aortic velocity corrected for HR; LVESP = Left Ventricle End Diastolic Pressure; +dP/dt = Maximum and Minimum First Derivative of the LV Pressure; LVW = Left Ventricular Weight; BW = Body Weight; TL = Tibial Length; SH = Sham-operated; AB = Aortic Banded; *p<0.01 compared with all other groups.
area (Fig. 1f). This eccentric ventricular remodeling was associated with upregulation of hypertrophic disease markers ANF and BNP (Fig. 1g and h) with no change in β-myosin heavy chain and α-skeletal muscle actin expression (data not shown). Consistent with established heart disease in Ace2<sup>−/y</sup> mutant mice, β-adrenergic agonist stimulation with dobutamine resulted in impaired chronotropic (change in HR: −45 ± 8 vs. 89 ± 11 bpm; p < 0.01; n = 7) and inotropic (change in VCF<sub>c</sub>: −1.43 ± 0.22 vs. 1.51 ± 0.13 circ/s; p < 0.01; n = 7) responses compared to littermate wild-type mice.

Ang II is known to mediate its pathological effects via the activation of AT1 receptors. To test this hypothesis, we treated young (8 weeks of age) Ace2<sup>−/y</sup> mice with the specific AT1 receptor blocker, irbesartan, until the age of 12 months. Long-term inhibition of AT1 receptors prevented the development of dilated cardiomyopathy at 6 months and
this protective effect persisted until 12 months of age based on echocardiographic and hemodynamic assessments (Fig. 2a and b, Table 1). Both morphometric assessment (Table 1) and ANF and BNP levels (Fig. 2c and d) showed a drastic reduction following AT1 blockade consistent with a marked reversal of pathological hypertrophy. Since Ace and Ace2 are predominantly expressed in the endothelium, we also examined myocardial capillary density. The vessel-to-nuclear ratio was 0.87±0.08 (n=4) in Ace2+/y and 0.95±0.13 in Ace2−/y mice (n=4; p=0.42) essentially ruling out hypovascularization of the myocardium in Ace2−/y mutant mice. These results demonstrated that the age-dependent

\[ \begin{align*}
\text{Fig. 2. Impaired heart function, increased pathological hypertrophy and myocardial oxidative stress in aged ACE2 null mice at 6 months of age were normalized} \\
\text{by long-term inhibition of AT1 receptors. (a–c) Pathological hypertrophy was normalized by irbesartan based on morphometry (a) (n=10) and expression of the} \\
\text{hypertrophy markers, ANF (b) and BNP (c) (n=8). (d–g) Increased myocardial oxidative stress as illustrated by increased NADPH oxidase activity (d) and} \\
\text{increased lipid peroxidation products, malondialdehyde (MDA) (e), hydroxynonenal (HNE) (f) and hexanal (HEX) (g) (n=10) were normalized in 6-month old} \\
\text{Ace2−/y mice treated with irbesartan. (h–i) Nitrotyrosine staining (h) and levels (i) showing increased nitrosylation in 6-month old Ace2−/y mice which was} \\
\text{prevented by irbesartan. \(*p < 0.05\) compared with all other groups. FS = Fractional Shortening; LVEDD = LV End Diastolic Dimension; LVW = LV Weight; TL =} \\
\text{Tibial Length; Irb = Irbesartan; PEG–SOD = polyethylene glycol–superoxide dismutase. \(*p < 0.01\) compared with all other groups.}
\end{align*} \]
cardiomyopathy in Ace2−/y mutant mice is mediated by Ang II activation of AT1 receptors.

3.2. AT1 blockade prevents oxidative stress and neutrophilic infiltration

Ang II is a key activator of reactive oxygen species (ROS) formation and oxidative stress in the cardiovascular system with the NADPH oxidase being a principal player [16,17,20,21]. Using the chemiluminescence assay, we found that NADPH oxidase activity was significantly increased in the LV of 6-month old Ace2−/y mice (Fig. 2e). Interestingly, AT1 blockade completely suppressed this increased NADPH oxidase activity in these mice consistent with the notion that Ang II-mediated activation of NADPH oxidase occurs via the AT1 receptors [16,17,20,21]. Both PEG–SOD and tiron normalized the increased superoxide production confirming that superoxide is the dominant ROS species in Ace2−/y hearts (Fig. 2e). Lipid peroxidation endproducts (aldehydes) and nitrosylation of proteins are sensitive markers of chronic myocardial oxidative damage. Myocardial aldehyde levels were indeed markedly increased in Ace2−/y mice and were normalized by chronic irbesartan treatment (Fig. 2f–h). In addition, immunohistochemistry showed a marked increase in nitrotyrosine staining (Fig. 2i and j), which was elevated by 3-fold in Ace2−/y cardiomyocytes and was normalized following chronic treatment with irbesartan (Fig. 2i and j).

Increased Ang II is also known to promote activation and infiltration of circulating neutrophils into tissues and activated neutrophils may also be a source of ROS [22,23]. Neutrophil-specific staining and quantitative analysis revealed a 4-fold increase in neutrophil infiltration in the myocardium of aged Ace2−/y mice (Fig. 3a and b) and with increased expression of inflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) (Fig. 3c–e) without changes in the expression of TNF-α, interferon-γ and iNOS (data not shown). Chronic AT1 blockade prevented neutrophil infiltration as
well as the increased expression of the proinflammatory cytokines, IL-1β, IL-6 and MCP-1 (Fig. 3c–e). Given the increased neutrophil infiltration and ventricular remodeling in aged Ace2−/y mice, we analyzed the expression of the neutrophil-specific collagenases, MMP-8 and MMP-13. There were 4-fold and 2-fold increases in myocardial MMP-8 (Fig. 3f) and MMP-13 (Fig. 3g) expression in Ace2−/y hearts, respectively, which were prevented by treatment with irbesartan. Macrophage staining showed no increase in macrophage infiltration in the Ace2−/y hearts or in expression of the macrophage-specific MMP, MMP-12 (data not shown). The activation of MAPK signaling pathways through GPCR signaling and oxidative stress mediates both adaptive and maladaptive responses in the heart [24]. In aged Ace2−/y mutant mice, there was increased phosphorylation of ERK1/2 and p38 pathways with no change in the JNK1/2 pathway (Fig. 4a–c). Consistent with the marked protection following irbesartan treatment, increased phosphorylation of the adaptive ERK1/2 pathway was maintained while preventing the phosphorylation and activation of the maladaptive MAPK pathways, JNK1/2 and p38 (Fig. 4a–c). The lack of suppression of phosphorylated ERK1/2 by AT1 blockade suggests that the ERK1/2 system is possibly activated by the cardioprotective AT2 receptors and/or other peptide systems. Collectively, these results indicate that chronic loss of ACE2 leads to AT1 receptor-mediated NADPH oxidase activation, increased oxidative stress, neutrophil recruitment and MAPK activation ultimately leading to adverse ventricular remodeling and heart disease.

3.3. Cardiac dysfunction in Ace2−/y mutant mice is critically dependent on PI3Kγ

Ang II binds and activates the GPCRs, AT1 and AT2 receptors and our data confirms that AT1 receptors are the dominant mediators of the adverse effects of Ang II. The downstream effects of GPCR activation are mediated primarily by the PI3Kγ isoform [14,25,26]. PI3Kγ is also a critical determinant of neutrophil function [27–29] and vascular superoxide production [30,31]. We used the

Fig. 4. Activation of the myocardial mitogen-activated protein kinase (MAPK) signaling pathways in aged Ace2−/y mutant mice at 6 months of age. (a) ERK1/2, JNK1/2 (b) and p38 (c) with the left column showing western blots and the right panel showing the quantitative analysis. In panel (a) the upper and lower fractions of each row refer to 44 and 42 kDa while in panel (b) the upper and lower fractions of each row refer to 55 and 46 kDa, respectively. AU = Arbitrary Unit; p = phosphorylated; IRB = irbesartan; ERK = Extracellular-Activated Kinase; JNK1/2 = c-Jun Activated Kinase. The upper panel refers to the phosphorylated kinase with lower panel reflecting the total protein level. n = 3 for each group; *p < 0.05 compared with all other groups.
Langendorff preparation to establish the critical role of PI3Kγ in mediating myocardial superoxide production in response to Ang II. Loss of p110γ, the catalytic subunit of PI3Kγ, leads to an 80% loss of myocardial superoxide production in response to a physiological dose of Ang II (30 nM) (Fig. 5a). Given the increased Ang II-dependent superoxide production and oxidative stress associated with loss of ACE2, we hypothesized that loss of PI3Kγ would minimize the cardiac effects of Ang II in Ace2−/− mutant mice. To test this hypothesis, we generated Ace2−/−/p110γ−/− double mutant mice. Consistent with the loss of superoxide production, chronic oxidative stress as assessed by myocardial malondialdehyde (MDA) level (b) was reduced oxidative stress as measured by myocardial malondialdehyde (MDA) level (b). (c–f) Loss of p110γ completely prevented the neutrophil infiltration into the myocardial tissue (Fig. 5d) and decreased expression of the cytokine, interleukin-1β (Fig. 5e), monocyte chemoattractant protein-1 (MCP-1) (Fig. 5f) and matrix metalloproteinase-8 (MMP-8) (g) with normalization of the degree of nitrotyrosine staining (h). n = 10 in all groups; *p < 0.01 compared with all other groups.

### Table 2

Loss of p110γ rescues the dilated cardiomyopathy in 6-month old Ace2−/− mutant mice

<table>
<thead>
<tr>
<th></th>
<th>Ace2+/y</th>
<th>Ace2−/−</th>
<th>p110γ+/y</th>
<th>Ace2−/− p110γ−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>519±12</td>
<td>512±9</td>
<td>531±17</td>
<td>524±13</td>
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<tr>
<td>PW (mm)</td>
<td>0.7±0.01</td>
<td>0.69±0.01</td>
<td>0.71±0.02</td>
<td>0.7±0.02</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.91±0.08</td>
<td>4.71±0.09*</td>
<td>3.87±0.07*</td>
<td>4.05±0.12</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.79±0.05</td>
<td>3.08±0.07*</td>
<td>1.76±0.06</td>
<td>2.02±0.08</td>
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<tr>
<td>FS (%)</td>
<td>54.6±1.7</td>
<td>36.1±2.2*</td>
<td>59.3±2.1</td>
<td>50.2±1.9</td>
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<tr>
<td>VCF (circ/s)</td>
<td>10.9±0.4</td>
<td>7.1±0.34*</td>
<td>12.9±0.35*</td>
<td>12.4±0.27</td>
</tr>
<tr>
<td>PAV (cm/s)</td>
<td>96.8±2.1</td>
<td>77.4±2.3*</td>
<td>115.6±2.6</td>
<td>109.6±3.2</td>
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<td>LVEDP (mm Hg)</td>
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<td>LVW/TL (mg/mm)</td>
<td>4.74±0.12</td>
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<td>4.69±0.13</td>
<td>5.23±0.19</td>
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</table>

See Table 1 for abbreviations; *p < 0.01 compared with all other groups; #p < 0.05 compared to the Ace2−/− group.

### 3.4. Phenotypic variation in aged Ace2−/− mutant mice

Our results provide unequivocal evidence that Ang II is a pivotal mediator of the cardiac dysfunction in aged Ace2−/− mutant mice. However, plasma and tissue levels of Ang II are variable possibly due to varying copies of the renin gene [32,33] and C57BL/6 mice have a low basal NADPH oxidase activity [34,35] and are resistant to myocardial inflammation [36] compared with other strains. Given the impact of genetic background on the phenotypic expression of genetic changes [37–39], we hypothesized that genetic background may modify the phenotype in aged Ace2−/− mutant mice. Heart Ang II levels were not elevated in Ace2−/− mice.
backcrossed into a pure C57BL/6 background after at least 8 generations compared with littermate wild-type (Ace2−/y) mice: 12.2 ± 2.6 vs 13.2 ± 3.9 fmol/mg protein (n = 7; p = 0.36) with no elevation in superoxide production: 89 ± 7.23 vs 95 ± 6.2 RLUs/mg protein (p = 0.41; n = 7). The lack of increased Ang II and oxidative stress in aged C57BL/6 Ace2−/y mice differs from our findings in aged Ace2−/y mice in a mixed background (see Figs. 2 and 3) [4]. Interestingly, as we outbred our Ace2−/y mice into a pure C57BL/6 background there was a gradual phenotypic drift such that the age-dependent changes in hypertrophy and cardiac function in aged Ace2−/y mice were less drastic after 3 generations of backcrossing and there was no measurable difference by the 8th generation of backcrossing (Table 3). These observations demonstrate that genetic background is a critical determinant of ACE2 action and confirms that Ang II-mediated activation of NADPH oxidase activity is an important mediator of heart disease.

4. Discussion

The monocarboxypeptidase, ACE2, which is predominantly expressed in the heart and kidneys shuttles angiotensin metabolism away from the formation of Ang II thereby functioning essentially as a negative regulator of the RAS [2]. In human heart failure, there is upregulation of ACE2 which may provide a key adaptive and cardioprotective mechanism [7–9]. Our current study shows that in the absence of ACE2, cumulative damage from Ang II acting via the AT1 receptors is the predominant cause of the age-dependent cardiomyopathy. The phenotypic alterations in our mouse models closely mimic the biochemical, structural and functional alterations in human heart failure. The AT1 receptor blocker, irbesartan, slowed the progression to heart failure in Ace2−/y mutant mice which is consistent with the use of AT1 receptor blockers as effective therapy for human heart failure. Consistent with the increased oxidative stress in the myocardium, loss of ACE2 also leads to Ang II-dependent renal oxidative damage and glomerular injury [6] which together with possible vascular injury may play a role in the overall age-dependent cardiomyopathy in Ace2 null mice.

We have shown that in aged Ace2−/y mice, increased Ang II action via AT1 receptors was associated with enhanced pathological hypertrophy, ventricular dilation and compromised systolic function. Ang II can induce reactive oxygen species such as H2O2 (hydrogen peroxide) and O2− (superoxide) formation via activation of NADPH oxidase [3,20,21,40]. Ang II-mediated activation of the NADPH oxidase complex via AT1 receptors has been well documented in cardiomyocytes, vascular and endothelial cells [17,21,40]. Reactive oxygen species interact with nitric oxide which may deplete endogenous nitric oxide [42] while also leading to the formation of reactive and toxic species such as peroxynitrite (ONOO−) [3,21,40]. NADPH oxidase activity is increased in human heart failure which may facilitate the negative inotropic effects of Ang II [41,43]. We observed a marked increase in Ang II-dependent infiltration of neutrophils into the myocardium in Ace2−/y deficient hearts which was markedly reduced by AT1 receptor blockade. Ang II mediates a wide variety of cardiac inflammatory processes and leads to upregulation of proinflammatory markers [22,23,44]. Ang II-mediated recruitment and activation of neutrophils perpetuate the free radical induced myocardial injury [20,45] and increase collagenase activity via the induction of MMP-8 expression which can degrade the extracellular matrix thereby compromising systolic function [18]. The activation of MAPK pathways is a characteristic finding in heart disease and plays a key role in the progression to heart failure [24]. Our data confirms the activation of myocardial MAPK pathways in aged Ace2−/y mutant mice and is consistent with changes observed following pressure-overload in young Ace2−/y mutant mice [19]. Indeed, ERK1/2, JNK1/2 and p38 pathways were all activated in Ace2−/y mutant mice and while AT1 blockade maintained the phosphorylation of the adaptive ERK1/2, activation of the maladaptive, JNK1/2 and p38, was selectively reduced.

The downstream effects of GPCR activation, such as the AT1 receptors, are mediated primarily by PI3Kγ [14,25,26,31]. PI3Kγ plays a key role in mediating agonist mediated stimulation in various tissue including neutrophils [27], myocardium [25] and vasculature [25,31]. Indeed, Ang II
infusion failed to promote vascular oxidative stress and superoxide production in \(p110\gamma^{-/-}\) mice [31]. Our data extends previous observations and shows that PI3K\(\gamma\) is a positive regulator of superoxide production in response to Ang II in the heart. Indeed, several of the subunits of the NADPH oxidase can be activated following PI3K\(\gamma\)-mediated generation of phosphatidylinositol-3,4,5-triphosphate (PIP\(_3\)) from its precursor phosphatidylinositol-4,5-bisphosphate (PIP\(_{2}\)) [31,46]. PI3K\(\gamma\) is also a key mediator of the activation of inflammatory cells including neutrophils especially in response to GPCR agonists [27–29]. Our data suggests that PI3K controls the recruitment of neutrophils into the heart in response to Ang II. Given that \(p110\gamma^{-/-}\) mice have increased basal myocardial contractility, we cannot rule a contributing role of this increased basal contractility in mediating the protection seen in \(Ace2^{+/+}/p110\gamma^{-/-}\) double mutant mice.

Our observation of the phenotypic variation of the impact of ACE2 was anticipated because of the varying levels of plasma and tissue Ang II levels in different mouse strains [32,33]. Indeed, mice of the Ola/129 background have been shown to carry two copies of the renin genes [32,33]. Consistent with this, we have previously shown that plasma and heart angiotensin I (1–10), the product of renin action on angiotensinogen, was increased in \(Ace2^{+/+}\) mice from an Ola/129 background [4]. Given the key pathophysiological role of Ang II, the lack of a discernable phenotype associated with a loss of ACE2 in a pure C57BL/6 background was predictable. Moreover, C57BL/6 mice which have low levels of expression and activity of NADPH oxidase are likely to be relatively resistant to the effects of Ang II [34,35,37]. Experimental conditions cannot be elicited as a possible explanation for this phenotypic variation as we have also clearly demonstrated the phenotypic variation in different mouse strains. More importantly, we have used an ex vivo technique (Langendorff preparation) to confirm the reduced basal myocardial contractility, we cannot rule a contributing role of this increased basal contractility in mediating the protection seen in \(Ace2^{+/+}/p110\gamma^{-/-}\) double mutant mice. Our findings suggest that strategies to enhance the action of ACE2 in the heart may serve as an important therapeutic intervention. Rescue experiments involving delivery of ACE2 lentivirus have shown that increasing ACE2 function is protective against cardiovascular disease in animal models [47,48]. Our data have provided important new insight into the pathophysiology of the RAS and ACE2 in heart failure and open up the possibility for new therapeutic avenues in the treatment of heart failure in humans. The phenotypic variations of ACE2 function in murine models have important implications for pharmacogenetics and disease association studies in humans. For example, polymorphic variations in the ACE2 gene have been linked to the development of pathological myocardial hypertrophy and heart disease in humans [12,13]. Variation in the human NADPH oxidase subunits including the p22 phox subunit [50] may interact with ACE2 polymorphisms to modify cardiovascular risk and disease progression in humans.

Acknowledgements

GYO is a clinician–scientist supported by the Heart and Stroke Foundation of Canada, Canadian Institute for Health Research and the TACTICS program. ZK is supported by the Heart and Stroke Foundation of Canada (PostDoctoral Fellowship) and the TACTICS program. JMP is funded by IMBA, Austrian Academy of Sciences and the Austrian National Bank.

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