Interaction of diabetes and ACE2 in the pathogenesis of cardiovascular disease in experimental diabetes

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ABSTRACT
Local and systemic AngII (angiotensin II) levels are regulated by ACE2 (angiotensin-converting enzyme 2), which is reduced in diabetic tissues. In the present study, we examine the effect of ACE2 deficiency on the early cardiac and vascular changes associated with experimental diabetes. Streptozotocin diabetes was induced in male C57BL6 mice and Ace2-KO (knockout) mice, and markers of RAS (renin–angiotensin system) activity, cardiac function and injury were assessed after 10 weeks. In a second protocol, diabetes was induced in male ApoE (apolipoprotein E)-KO mice and ApoE/Ace2-double-KO mice, and plaque accumulation and markers of atherogenesis assessed after 20 weeks. The induction of diabetes in wild-type mice led to reduced ACE2 expression and activity in the heart, elevated circulating AngII levels and reduced cardiac Ang-(1–7) [angiotensin-(1–7)] levels. This was associated structurally with thinning of the LV (left ventricular) wall and mild ventricular dilatation, and histologically with increased cardiomyocyte apoptosis on TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining and compensatory hypertrophy denoted by an increased cardiomyocyte cross-sectional area. By contrast Ace2-KO mice failed to increase circulating AngII concentration, experienced a paradoxical fall in cardiac AngII levels and no change in Ang-(1–7) following the onset of diabetes. At the same time the major phenotypic differences between Ace2-deficient and Ace2-replete mice with respect to BP (blood pressure) and cardiac hypertrophy were eliminated following the induction of diabetes. Consistent with findings in the heart, the accelerated atherosclerosis that was observed in diabetic ApoE-KO mice was not seen in diabetic ApoE/Ace2-KO mice, which experienced no further increase in plaque accumulation or expression in key adhesion molecules beyond that seen in ApoE/Ace2-KO mice. These results point to the potential role of ACE2 deficiency in regulating the tissue and circulating levels of AngII and their sequelae in the context of diabetes, as well as the preservation or augmentation of ACE2 expression or activity as a potential therapeutic target for the prevention of CVD (cardiovascular disease) in diabetes.

Key words: angiotensin, angiotensin-converting enzyme 2, diabetes, diabetic cardiomyopathy, renin–angiotensin system.
Abbreviations: ACE, angiotensin-converting enzyme; AngI etc., angiotensin I etc.; Ang-(1–7), angiotensin-(1–7); ApoE, apolipoprotein E; AT1 receptor, AngII type 1 receptor; AT2 receptor, AngII type 2 receptor; BNP, brain natriuretic peptide; BP, blood pressure; CVD, cardiovascular disease; IL, interleukin; KO, knockout; LV, left ventricular; LVEDD, LV diameter at end-diastole; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; MMP-8, matrix metalloproteinase-8; RAS, renin–angiotensin system; RT–PCR, reverse transcription–PCR; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; VCAM, vascular cell adhesion molecule.
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INTRODUCTION

Diabetes is associated with increased cardiovascular morbidity and mortality [1]. Chronic hyperglycaemia leads to both accelerated atherosclerosis and direct cardiac injury that result from the activation of a complex range of pathogenic pathways. Although the pathogenesis of diabetes-associated CVD (cardiovascular disease) is multi-factorial, central among these mediators is activation of the RAS (renin–angiotensin system) in the heart [2–4] and in the vasculature [5]. In particular, it is known that AngII (angiotensin II) has a number of direct pro-atherosclerotic effects [6–8], whereas blockade of the RAS has anti-atherosclerotic actions [5,9], additional and independent of systemic BP (blood pressure) [5]. Similarly, AngII-dependent signalling in the diabetic heart is associated with oxidative damage, fibrogenesis and myocyte apoptosis [2–4]. The primacy of the RAS in the development and progression of cardiovascular pathology associated with diabetes is supported by findings in a number of different models in which blockade of the RAS was able to attenuate or prevent cardiac damage [10,11] and atherosclerotic plaque accumulation [5,9,12], independent of BP-lowering.

Traditionally, research interest has focused on inhibiting the synthesis of AngII and/or preventing activation of the AT1 receptor (AngII type 1 receptor). However, pathways that regulate the degradation of AngII are also important for balancing levels of AngII with other Ang peptides. In the murine cardiovascular system, the carboxypeptidase activity of ACE2 (angiotensin-converting enzyme 2) is the primary pathway for the metabolism of AngII [13,14], which it converts into Ang-(1–7) (angiotensin-1–7). Genetic deficiency of ACE2 results in increased tissue and circulating levels of AngII [15,16], whereas Ang-(1–7) levels are reduced [17]. These changes are associated with progressive cardiac dysfunction [15] and accelerated atherosclerosis [18] in susceptible strains. Our group has shown previously that ACE2 gene expression is significantly reduced in diabetic eye and kidney [19,20]. We hypothesize that this reduction in ACE2 plays a significant role in the development of pathological changes in the heart and vasculature associated with experimental diabetes. In the present study, we explore the early vascular and cardiac changes associated with diabetes, in the presence and absence of ACE2.

MATERIALS AND METHODS

Animal models
C57BL6 and ApoE (apolipoprotein E)-KO (knockout) mice (on a C57BL6 background) were sourced from the Precinct Animal Centre of the Baker IDI Heart and Diabetes Institute, as described previously [21]. Ace2-KO mice (also on a C57BL6 background) were kindly provided by Professor Josef Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria), and generated as described previously by his group [15]. To generate ApoE/Ace2-KO mice, single KO strains were back-crossed ten generations, with genetic identity confirmed by tail genotyping at each generation [18].

In these studies, male mice were used from four groups: (i) C57BL6, (ii) Ace2-KO, (iii) ApoE-KO and (iv) ApoE/Ace2-double KO (n = 10 per group), with all animals on the same C57BL6 background. At 10 weeks of age and weighing between 20–25 g, mice from each group were randomly allocated to receive streptozotocin (55 mg/kg of body weight; Sigma) or buffer (sodium citrate buffer, pH 4.5) delivered intraperitoneally in five consecutive daily doses. This regimen induces an insulinopenic form of diabetes associated with hyperglycaemia (blood glucose ∼30 mM) but with sufficient β-cell function to prevent ketosis or require insulin supplementation. The presence of diabetes was confirmed by a fasting blood glucose level >15 mM 1 week after the first dose of streptozotocin. Throughout the study animals were given access to standard mouse chow (Animal Resources Centre) and water ad libitum.

All experiments were approved by the animal ethics committee of the Alfred Medical Research Precinct and conform 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).

Radiotelemetry studies
The Ace2-KO mouse is associated with mild-to-moderate hypertension when bred on a C57BL6 background [22], as opposed to other mouse strains [15,22,23]. To better characterize BP control in this specific model and its response to diabetes, 10-week-old wild-type male C57BL6 mice and Ace2-KO mice were implanted with telemetry devices (TA11PA-C20; DataSciences International) under halothane open circuit anaesthesia to monitor MAP (mean arterial pressure). The catheter tip was placed in the aortic arch via the carotid artery, and the transmitter was placed under the skin at the flank. After allowing 10 days to recover, BP recordings were collected in each group over a 72 h period by a receiver (model RPC-1; Data Sciences International) passed to an analogue converter (model RP11A; Data Sciences International) and average daily BP tracings generated as described previously [24]. Streptozotocin diabetes was randomly induced as detailed above and further recordings taken after 1 week of diabetes.

Echocardiographic studies
In vivo LV function and chamber dimensions was assessed with two-dimensional targeted M-mode and colour directed Doppler echocardiography after 10 weeks of diabetes (20 weeks old), while mice were under
light anaesthesia (ketamine at 60 mg/kg of body weight, xylazine at 15 mg/kg of body weight and atropine at 0.45 mg/kg of body weight, intraperitoneal) using a Hewlett-Packard Sonos 5500 ultrasonograph with a 15 MHz linear array transducer as described previously [25,26]. This time point was selected as it represents the earliest time-point that we are able to observe significant functional and structural changes associated with diabetes. Images were analysed off-line in a blind fashion.

**Plaque area quantification**

To determine the effects of ACE2 on diabetes-associated atherosclerosis, apoE-KO mice were monitored for 20 weeks, a time point associated with quantifiable plaque accumulation in all parts of the aorta. At this time, all mice were killed using an intraperitoneal injection of Euthal (10 mg/kg of body weight; Delvet), followed by exsanguination via cardiac puncture. In half of the animals in each group, aortae were collected and placed in 10 % neutral-buffered formalin and quantified for lesion area before being processed for subsequent immunohistochemical analysis. In the other half, aortae were snap-frozen in liquid nitrogen and stored at –70 °C for subsequent RNA extraction.

Plaque area was quantified as described previously [9]. In brief, aortae removed from mice were cleaned of excess fat under a dissecting microscope and subsequently stained with Sudan IV–Herxheimer’s solution (0.5 %) (Gurr). Aortae were dissected longitudinally, divided into arch, thoracic and abdominal segments, and pinned flat on to wax. Images were acquired with a dissecting microscope via cardiac puncture. In half of the animals in each group, aortae were collected and placed in 10 % neutral-buffered formalin and quantified for lesion area before being processed for subsequent immunohistochemical analysis. In the other half, aortae were snap-frozen in liquid nitrogen and stored at –70 °C for subsequent RNA extraction.

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**Quantitative real-time PCR**

Gene expression of markers of atherosclerosis, cardiac dysfunction and various components of the RAS, including ACE, ACE2, prorenin, AT1a receptor, AT2 receptor (AngII type 2 receptor) and chymase-1, were also determined by real time quantitative RT–PCR (reverse transcription–PCR) in aortic and cardiac homogenates [27]. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; PerkinElmer) as utilized previously by our group [27]. Gene expression was normalized to 18S mRNA and reported as ratios compared with the level of expression in untreated control mice, which were given an arbitrary value of 1.

**Activity of the intra-cardiac and circulating RAS**

Cardiac ACE2 activity was determined as previously described using the intra-molecularly quenched synthetic ACE2-specific substrate o-aminobenzoic acid-Ser-Pro-Tyr(NO2)-OH (Peptides International) [20]. The plasma and intra-cardiac AngII concentrations were measured by a commercial radioimmunoassay (ProSearch International) utilizing an selective polyclonal antibodies to AngII, Ang-(1–7) and 125I-AngII. Before analysis, snap frozen tissue samples were homogenized in ice-cold methanol in the presence of protease inhibitors (50 mmol/l EDTA, 0.5 mmol/l o-phenanthroline, 1 mmol/l N-ethylmaleimide and 0.1 mmol/l pepstatin A). Blood samples were collected on ice into the same cocktail and then rapidly processed. Tissue samples were centrifuged to remove protein, and the upper (aqueous) phase was removed and lyophilized in glass tubes at –80 °C. Results are expressed adjusted for the wet cardiac weight.

**Immunohistochemistry and ELISA**

Paraffin serial sections 4-μm thick were prepared from 4 % PFA (paraformaldehyde)-fixed paraffin-embedded mouse aorta and hearts. Cardiomyocyte hypertrophy was assessed by measuring cross-sectional area of 100 cardiomyocytes in the left ventricle near the endocardial region, assessing only those with nearly circular capillary profiles. For immunohistochemistry, sections were de-waxed and hydrated, then endogenous peroxidase was quenched for 20 min using 3 % H2O2 in PBS. To localize ACE2, a rabbit polyclonal anti-ACE2 521 antibody at a dilution of 1:250 raised against ACE2 residues 489–508 [28] was used (antibody donated by Millennium Pharmaceuticals). Specific staining was detected using the standard ABC (avidin–biotin complex) method [9]. Sections were lightly counter-stained with haematoxylin and eosin. Non-specific staining was tested with 1 % non-immunized goat serum. Images were acquired and quantified on an Olympus BX50 microscope using Optimis (version 6.2) and digitized using a colour video camera (three-charge-coupled device; JVC). Soluble VCAM (vascular cell adhesion molecule) was measured in plasma samples using a commercial ELISA (R&D Biosystems) and performed according to manufacturer’s instructions.

**Statistical analysis**

Continuous data are expressed as means ± S.E.M. Differences in the mean among groups were compared using two-way ANOVA with diabetic status and ACE2 status as the two variables. Pairwise multiple comparisons were made using the Student–Newman–Keuls post-hoc analysis to detect significant differences among groups. P < 0.05 was considered statistically significant.
Table 1  General and echocardiographic data in 20-week-old C57BL6 and Ace2-KO mice animals with and without 10 weeks of diabetes (n = 6 per group)

Results show means ± S.E.M. LVESD, LV end-diastolic/systolic dimensions; LVW, LV weight; BW, body weight; LVPW(d/s), LV posterior wall (diastolic/systolic); *P < 0.05 compared with control C57BL6 mice; ‡P < 0.05 compared with control Ace2-KO mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C57BL6 mice</th>
<th>Ace2-KO mice</th>
<th>Diabetes + C57BL6 mice</th>
<th>Diabetes + Ace2-KO mice</th>
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</thead>
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<tr>
<td>Body weight (g)</td>
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<td>31 ± 1</td>
<td>26 ± 1*</td>
<td>26 ± 1*‡</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
<td>29 ± 1*</td>
<td>29 ± 1*</td>
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<tr>
<td>Glycated haemoglobin (%)</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>15 ± 1*</td>
<td>15 ± 1*</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120 ± 3</td>
<td>126 ± 2*</td>
<td>114 ± 5</td>
<td>118 ± 4§</td>
</tr>
<tr>
<td>Mean myocyte diameter (μm)</td>
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<td>20.5 ± 0.7*</td>
<td>21.5 ± 0.8*</td>
<td>21.1 ± 0.9*</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
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<td>4.1 ± 0.2*</td>
<td>3.0 ± 0.2*</td>
<td>3.1 ± 0.3§</td>
</tr>
<tr>
<td>LVPWd</td>
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<td>0.46 ± 0.03*</td>
</tr>
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<td>1.08 ± 0.02</td>
<td>1.08 ± 0.04</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>LVEDD (mm/g BW)</td>
<td>0.14 ± 0.01</td>
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<td>0.17 ± 0.01*</td>
<td>0.18 ± 0.01*</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>29 ± 7</td>
<td>29 ± 5</td>
<td>40 ± 4*</td>
<td>40 ± 7‡</td>
</tr>
</tbody>
</table>

Figure 1  MAP in C57BL6 and Ace2-KO mice, with and without diabetes, as measured by 72 h radiotelemetry (n = 6 per group)

*Compared with control; P < 0.05.

RESULTS

BP levels

Ace2-KO mice had a modestly higher MAP (mean arterial pressure) when compared with C57BL6 mice as measured by radiotelemetry (Figure 1). The induction of diabetes had a small but significant haemodynamic effect in C57BL6 mice (P < 0.001 compared with control). Diabetes in Ace2-KO mice also resulted in a fall in MAP, which was proportionally greater in Ace2-KO mice than in C57BL6 animals (P < 0.01), such that the difference in BP between C57BL6 and Ace2-KO mice was eliminated in diabetic mice (Figure 1). These BP differences were not significantly different from those obtained using tail-cuff plethysmography as later time points (e.g. Table 1).

ACE2 expression and activity

In the heart, ACE2 expression was greatest in the cardiac vessels and endocardium, with more limited expression in cardiac myocytes (Figure 2 and Supplementary Figure S1 at http://www.clinsci.org/cs/123/cs1230519add.htm). The induction of diabetes was associated with reduced ACE2 expression and activity in the heart (Figure 2) and the vasculature (Figure 2) in C57BL6 mice. No ACE2 expression or activity was observed in Ace2-KO mice (results not shown).

Angiotensin peptide levels in the circulation and the heart

The induction of diabetes in C57BL6 mice was associated with a significant increase in circulating AngII when compared with control mice (Figure 3). This diabetes-associated increase in circulating AngII was not observed in Ace2-KO mice. However, baseline AngII levels in Ace2-KO mice were approximately twice than those seen in wild-type mice (Figure 3).

Cardiac AngII levels were elevated in Ace2-KO mice; however, in contrast with plasma levels of AngII, the intra-cardiac concentration of AngII was not significantly modified following the induction of diabetes in C57BL6 mice when compared with non-diabetic controls (Figure 3). By contrast, the induction of diabetes in Ace2-KO mice was associated with a paradoxical reduction in intra-cardiac AngII levels (Figure 3).

In addition to degrading AngII, ACE2 is also a major source of Ang-(1–7). Ace2-KO mice had reduced circulating and tissue levels of Ang-(1–7). Diabetes was also associated with reduced levels of Ang-(1–7).
Figure 2  ACE2 expression and activity in the heart (A, C) and aorta (B, D) of C57BL6 mice with and without 10 weeks of diabetes
White bar denotes control mice and black bar denotes mice with diabetes ($n = 6$ per group). Ace2-KO mice showed no activity and no immunostaining (results not shown) $^*P < 0.05$ compared with control.

Figure 3  Effect of ACE2 deficiency (shaded bars) and diabetes on AngII (A), Ang-(1–7) (B) and Ang I (C) levels in the heart and AngII in the circulation (D) ($n = 6$ per group)
$^*P < 0.05$ compared with control; $^#P < 0.05$ compared with non-diabetic Ace2-KO mice. D, diabetes.

However, the induction of diabetes in Ace2-KO mice did not reduce Ang-(1–7) levels beyond that observed in non-diabetic Ace2-KO mice (Figure 3). In addition, intra-cardiac AngI (angiotensin I) levels were unchanged following the induction of diabetes in Ace2-KO mice, whereas they were lower in diabetic wild-type mice when compared with their C57BL6 controls (Figure 3).

To explore further whether this phenomenon may be due to differences in cardiac Ang synthesis and or degradation, the differential expression of alternative...
regulators of the AngII levels was also measured. Notably, the expression of the novel angiotensinase, MMP-8 (matrix metalloproteinase-8) was reduced following the onset of diabetes in both C57BL6 and Ace2-KO mice (Table 2). However, the cardiac expression of chymase-1, which represents an alternative source of AngII in the heart, was increased in the diabetic heart from C57BL6 mice, but not diabetic Ace2-KO mice (Table 2). The expression of cardiac ACE was not modified by diabetes in wild-type or Ace2-KO mice and there were no significant changes in the gene expression of other cardiac angiotensinases, including prorenin, neutral endopeptidase or prolyl endopeptidase expression in either diabetic or Ace2-KO mice (results not shown).

Diabetes was also associated with an increase in the gene expression of the AT1 and AT2 receptors in C57BL6 mice. By contrast, the induction of diabetes in Ace2-KO mice was associated with an increase in only the AT1 receptor, whereas expression of the AT2 receptor remained unchanged (Table 2).

### Structural and functional changes in the diabetic heart

To examine the role of ACE2 in early cardiac changes in streptozotocin diabetes, echocardiograms were performed after 10 weeks of diabetes, the earliest time point at which structural changes are observed. As described previously in this model [11], the adjusted LV mass was reduced by diabetes when compared with non-diabetic controls. In wild-type mice, this was associated with modest thinning of the LV wall and mild ventricular dilatation, denoted by a decrease in the LVPW (LV posterior wall) and increase in the LVEDD (LV diameter at end-diastole) respectively (Table 1). However, the mean cross-sectional area of cardiomyocytes was increased following the induction of diabetes in wild-type mice, consistent the known effects of apoptosis and compensatory hypertrophy seen in this experimental model [11].

Ace2-KO mice also had increased weight-adjusted cardiac mass (by 30–35%) when compared with wild-type C57BL6 mice. This was associated with modest ventricular hypertrophy and dilatation when compared with wild-type C57BL6 mice, as detected by echocardiographic measurements at 20 weeks of age and by an increase in the mean cross-sectional area of cardiomyocytes (Table 1). These structural changes were not associated with abnormal systolic performance or significant diastolic stiffening (results not shown).

The induction of diabetes in Ace2-KO mice also resulted in a significant reduction in total cardiac and LV mass, again eliminating the difference between (Ace2-replete and -deficient) strains (P = 0.9). In contrast with wild-type mice, diabetes was not associated with additional increase in LVEDD in Ace2-KO mice or in the mean cross-sectional area of cardiomyocytes, beyond that seen in the non-diabetic state (Table 1).

### ACE2 and diabetes associated cardiac apoptosis

These early changes in the diabetic heart are thought to be partly mediated by (AngII-dependent) myocyte apoptosis, which is maximal 1 week after the onset of hyperglycaemia and precedes the ventricular thinning [11]. In our present study, after 1 week of diabetes TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining for apoptosis was increased in C57BL6 mice following the onset of diabetes in C57BL6 mice (Figure 4).

Subsequent compensatory cardiac hypertrophy in diabetes, denoted by increased cardiomyocyte diameter (Table 1), was associated with reactivation of the fetal gene programme, characterized by a switch to β-MHC (β-myosin heavy chain) expression in the adult murine heart. Similarly, other markers of cardiac damage and dysfunction, including BNP (brain natriuretic peptide), osteopontin [29] and CTGF (connective tissue growth factor) [30], were increased in wild-type diabetic mice (Table 3). However, further activation of fetal gene expression, apoptosis and expression of markers of cardiac remodelling were unaltered following the
induction of diabetes in Ace2-KO mice when compared with non-diabetic Ace2-KO mice (Table 3).

Aortic plaque area
Owing to very efficient lipoprotein metabolism in mice, a pro-atherogenic phenotype is required for the development of an atherosclerotic plaque. In this study, we used the ApoE-KO mouse, the model most widely employed to investigate experimental atherosclerosis associated with diabetes, with plaque accumulation and morphology in this strain resembling human disease [5,9]. As previously described [18], the total plaque area, quantified as a percentage area of aorta stained red with Sudan IV, was significantly increased in ApoE/Ace2-double-KO mice when compared with ApoE-KO mice. Similarly, the induction of diabetes was also associated with increased plaque accumulation. However, the combination of ACE2 deficiency and diabetes did not increase plaque accumulation beyond that observed in non-diabetic ApoE/Ace2-double-KO mice or diabetic ApoE-KO mice alone (Figure 5). No plaque was observed in C57BL6 and Ace2-KO mice in the absence of genetic susceptibility conferred by the ApoE gene deletion (results not shown).

Expression of pro-atherogenic mediators
Diabetes for 20 weeks in ApoE-KO mice was associated with a marked up-regulation in the expression of pro-atherogenic mediators, including VCAM-1, IL-6 (interleukin-6) and MCP-1 (monocyte chemoattractant protein-1) when compared with non-diabetic ApoE-KO controls (Figure 6). In addition, the expression of ACE2 was reduced in the aorta, whereas ACE, Mas-1 and AT1 receptor gene expression were increased, as observed in the diabetic heart (see above). By contrast, the induction of diabetes in ApoE/Ace2-double-KO mice did not further increase the gene expression of pro-inflammatory mediators, beyond that seen in non-diabetic-double-KO controls (Figure 6). In addition, the circulating concentration of VCAM was not increased in Ace2-KO mice following the onset of diabetes (ApoE/Ace2-double-KO mice, 0.32 ± 0.08; diabetic ApoE/Ace2-double-KO mice, 0.32 ± 0.08) when compared with a 2-fold increase observed in diabetic ApoE-KO mice (ApoE-KO mice, 0.22 ± 0.06; diabetic ApoE-KO mice, 0.46 ± 0.06; P < 0.001).

DISCUSSION
Activation of the RAS is a key initiator of vascular injury and cardiac dysfunction associated with diabetes [2–5,9–12]. It is thought that increased circulating levels of AngII, in combination with increased expression and density of the angiotensin receptors in the heart and vasculature [11], potentially leads to increased AngII-dependent signalling and its downstream sequelae including apoptosis, compensatory hypertrophy and inflammation. In this context, down-regulation of ACE2 expression and activity in diabetes potentially plays a role, both in elevating AngII in the circulation and in inappropriately maintaining AngII levels and reducing Ang-(1–7) in the heart in the context of RAS activation. This is suggested by our finding that these phenomena were not observed in Ace2-KO mice. We hypothesize

Table 3 Gene expression of components of markers of cardiac hypertrophy and fibrosis in the heart, as measured by real time RT—PCR

Relative gene expression is shown. *P < 0.05 compared with control C57BL6 mice; †P < 0.05 compared with diabetic C57BL6 mice. ANP, atrial natriuretic peptide; TNFα, tumour necrosis factor α.

<table>
<thead>
<tr>
<th>Gene</th>
<th>C57BL6 mice</th>
<th>Ace2-KO mice</th>
<th>Diabetes + C57BL6 mice</th>
<th>Diabetes + Ace2-KO mice</th>
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<td>β-MHC</td>
<td>1.0</td>
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<td>1.5 ± 0.2*</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>ANP</td>
<td>1.0</td>
<td>1.1 ± 0.1</td>
<td>1.7 ± 0.3†</td>
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</tr>
<tr>
<td>BNP</td>
<td>1.0</td>
<td>1.5 ± 0.2*</td>
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<tr>
<td>TNFα</td>
<td>1.0</td>
<td>1.6 ± 0.2*</td>
<td>1.8 ± 0.3†</td>
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<tr>
<td>CTGF</td>
<td>1.0 ± 0.2</td>
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<td>Osteopontin</td>
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</tr>
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</table>

Figure 4 Apoptosis and dysfunction in the diabetic heart
Mean number of TUNEL positive cells per field counted over ten cardiac fields in wild-type (solid) and Ace2-KO mice (cross hatch) in the presence (shaded bars) or absence of 1 week of diabetes (n = 6 per group). No differences were observed at later time points (results not shown). *P < 0.05 compared with control; †P < 0.05 compared with non-diabetic Ace2-KO mice. D, diabetes.
that, by being unable to modify their Ace2 expression, Ace2-KO mice were unable to develop some of the RAS-dependent diabetes-associated vascular and cardiac changes beyond those seen in non-diabetic Ace2-KO mice. Indeed, the major phenotypic differences between Ace2-deficient and Ace2-replete mice with respect to BP, cardiac hypertrophy and accelerated atherosclerosis were eliminated following the induction of diabetes. As such, these results point to a role for ACE2 in regulating the levels of AngII and their pathological sequelae in the context of diabetes.

The factors controlling AngII levels in the systemic circulation have been traditionally thought to be different from those performing this action locally, particularly within the heart. For example, ACE-deficient mice have reduced circulating AngII, while cardiac levels are not significantly modified [31]. As ACE inhibitors are able to prevent diabetes-associated cardiac dysfunction [11,32],
it was initially proposed that much of their beneficial effect was therefore mediated by their systemic actions. This also led to the suggestion that other (upstream) angiotensinases, such as chymase-1 and MMP-8, may be more important sources of AngII in the heart [31]. By contrast, we show that Ace2-KO mice have increased levels of AngII both in their tissues and bloodstream, which potentially contribute to modestly increased systolic BP and cardiac enlargement in these mice (Table 1). These data suggest that factors such as ACE2, which regulate the degradation of AngII, may be equally or more important than enzymes that regulate AngII synthesis, such as ACE, in determining the cardiac balance of the RAS, particularly in the context of diabetes.

ACE2 is the major enzyme that degrades AngII to form Ang-(1–7) in the vasculature and the heart. Consequently, alongside elevated levels of AngII, deficiency of ACE2 also leads to reduced circulating and tissue levels of Ang-(1–7). This may have important consequences for the pathogenesis of CVD, as Ang-(1–7) is widely regarded as an indirect antagonist for AngII-mediated signalling, as well as having vaso- and cardio-protective actions in its own right [33,34]. Certainly, an infusion of Ang-(1–7) is cardioprotective in diabetic rats [35] and atheroprotective in ApoE-KO mice [36]. It is possible to speculate that the imbalance of increased tissue and circulating levels of AngII alongside reduced synthesis of Ang 1–7, contributes to the observed pro-inflammatory and pro-atherogenic phenotype associated with Ace2 deficiency, as well as that associated with diabetes. The actions of Ang-(1–7) are partly mediated via activation of the Mas-1 receptor [37,38], which has the ability to directly trigger cardioprotective pathways [33,34] as well as hetero-oligomerize with the angiotensin receptors and antagonize AngII-dependent signalling [39]. In our diabetic mice, the vascular expression of Mas receptor was increased, both in the presence and absence of Ace2 deficiency. This may partly be in response to reduced levels of Ang-(1–7). However, additive effects on Ang-(1–7) were not observed in diabetic Ace2-KO mice, suggesting that other factors may induce also Mas-1 expression in diabetes. While we propose that one reason why Ace2-KO mice may be partially protected from the effects of diabetes is due to the lack of induction of cardiac AT1 receptor and reduced cardiac AngII levels following the onset of diabetes, the mechanisms responsible for such differential changes remain to be established. It is possible to speculate that in wild-type mice, diabetes-associated reductions in ACE2 expression and activity may be offset by other changes in RAS homeostasis, including angiotensin-receptor-dependent endocytosis or other angiotensinase activity. However, in Ace2-KO mice, where ACE2 expression cannot change, but the expression of other RAS components can, the net result of diabetes appears to be a fall in intra-cardiac AngII levels. Certainly, the increase in AT1 receptor expression observed associated with diabetes in wild-type heart was not observed in diabetic Ace2-KO mice. The expression of chymase-1, which represents an alternative source of AngII in the heart, was also increased in the diabetic heart from wild-type mice, but not from diabetic Ace2-KO mice (Table 2). In addition, expression of the novel angiotensinase, MMP-8 was also reduced following the onset of diabetes in both wild-type and Ace2-KO mice.

As MMP-8 deficiency is associated with reduced tissue levels of AngII [40], it is conceivable that the diabetes-associated decline in MMP-8 both contributes to both the paradoxical fall in Ace2-KO mice following the onset of diabetes and the failure of AngII levels to increase in wild-type mice following the onset of diabetes, despite a significant reduction in ACE2 expression and activity in the diabetic heart.

The present study specifically examined the early changes associated with Type 1 (streptozotocin) diabetes in mice bred on a C57BL6 background. These changes typically include reduced LV mass and thinning of the ventricular wall [3,4], which are thought to reflect (AngII dependent) myocyte apoptosis [11]. This is associated with compensatory cardiac hypertrophy manifested in an increase in cardiomyocyte diameter and increased expression of fetal cardiomyocyte markers such as MHC, similar to that observed in clinical diabetes. However, his model is not associated with myocardial fibrosis and diastolic dysfunction that may be observed in longer-term studies and/or in more susceptible strains, as well as in human diabetic cardiomyopathy.

Activation of the RAS is known to contribute to the development and progression of atherosclerosis in the context of diabetes. Our group and others have shown previously that both ACE inhibition and blockade of the AT1 receptor are able to reduce atherosclerosis in diabetic ApoE-KO mice [5,9,12], independent of BP-lowering effects. In the diabetic vasculature, ACE2 expression and activity is reduced [21]. In this context we hypothesize that, as in the heart, this reduction inappropriately sustains RAS activation and facilitates atherogenesis. Consistent with this hypothesis, we have recently shown that genetic deletion of Ace2, encoding the major enzyme that degrades AngII in the vasculature, significantly increases plaque accumulation in ApoE-KO mice [18]. Similarly, there is increased atherogenesis following an infusion of AngII [6]. However, in the present study, ApoE/Ace2-double-KO mice did not accumulate additional plaque following the induction of diabetes beyond that seen in non-diabetic ApoE/Ace2-double-KO mice. This may be partly explained by the fact that the induction of diabetes was unable to modify ACE2 expression in these mice, and therein, execute activation of the RAS and its subsequent enhancement of atherosclerosis. Such findings potentially point to ACE2 as an important and novel target for vascular-protective therapies. Indeed, recent studies...
suggest that ACE2 delivered as an adenovirus may, in part, attenuate atherosclerotic in Apoe−/− mice [41]. The mouse genetic background significantly alters the cardiovascular phenotypes in response to both ACE2 deficiency and to diabetes [22,23]. This may partly explain why we were able to demonstrate both early cardiac hypertrophy and modestly increased BP in Ace2−/− mice, while the original reports by Crackower et al. [15] described only thinning of the LV wall without hypertrophy or hypertension in animals of a mixed background, despite both strains demonstrating elevated levels of tissue and circulating AngII. More recent reports also confirm the finding of hypertension [22] and early hypertrophy in Ace2−/− mice [23]. The underlying explanation for these differences remains to be established. The original description also reported a modest increase in cardiac AngII, while we saw a decline in AngI levels, which may have partly offset the effects of ACE2 in our model. Such divergent findings potentially imply that ACE2 deficiency requires a susceptible phenotype for its full expression. In the same way, diabetes-associated cardiac injury also requires additional susceptibility for its full effects to be realized. For example, following the induction of diabetes, BP differences between wild-type and Ace2−/− mice were lost. Although this may seem paradoxical, we speculate that, in the absence of Ace2 (a key substrate for diabetes effects), AngII failed to increase in the circulation following the onset of diabetes (Figure 3), and with it, its effects of cardiac and vascular pathology are partly attenuated. In summary, diabetes is associated with reduced expression and activity of ACE2. This potentially contributes to increased levels of circulating AngII and the inappropriate persistence of cardiac AngII levels in the presence of increased AT1 receptor expression and activation. This in turn contributes to diabetes-associated cardiac damage and augmented atherosclerosis. These findings point to the preservation or augmentation of ACE2 expression or activity as a potential therapeutic strategy for the prevention of CVD, particularly in the context of diabetes.

AUTHOR CONTRIBUTION

Chris Tikellis, Raelene Pickering and Despina Tsorotes researched the data and contributed to the discussion. Xiao-Jun Du and Helen Kiriazis performed the echocardiography, Thu-Phuc Nguyen-Huu and Geoffrey Head performed the telemetry, Mark Cooper edited the paper and contributed to the discussion, and Merlin Thomas designed the study and wrote the paper.

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ACE2 and the diabetic heart


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Interaction of diabetes and ACE2 in the pathogenesis of cardiovascular disease in experimental diabetes

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Figure S1  Immunostaining for ACE2 in the heart of C57BL6 mice with and without 10 weeks of diabetes
Ace2-KO mice showed no immunostaining (results not shown).