Angiotensin-converting enzyme 2 regulates renal atrial natriuretic peptide through angiotensin-(1–7)

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ABSTRACT

Deficiency of ACE2 (angiotensin-converting enzyme 2), which degrades Ang (angiotensin) II, promotes the development of glomerular lesions. However, the mechanisms explaining why the reduction in ACE2 is associated with the development of glomerular lesions have still to be fully clarified. We hypothesized that ACE2 may regulate the renoprotective actions of ANP (atrial natriuretic peptide). The aim of the present study was to investigate the effect of ACE2 deficiency on the renal production of ANP. We evaluated molecular and structural abnormalities, as well as the expression of ANP in the kidneys of ACE2-deficient mice and C57BL/6 mice. We also exposed renal tubular cells to AngII and Ang-(1–7) in the presence and absence of inhibitors and agonists of RAS (renin–angiotensin system) signalling. ACE2 deficiency resulted in increased oxidative stress, as well as pro-inflammatory and profibrotic changes. This was associated with a down-regulation of the gene and protein expression on the renal production of ANP. Consistent with a role for the ACE2 pathway in modulating ANP, exposing cells to either Ang-(1–7) or ACE2 or the Mas receptor agonist up-regulated ANP gene expression. This work demonstrates that ACE2 regulates renal ANP via the generation of Ang-(1–7). This is a new mechanism whereby ACE2 counterbalances the renal effects of AngII and which explains why targeting ACE2 may be a promising strategy against kidney diseases, including diabetic nephropathy.

INTRODUCTION

The RAS (renin–angiotensin system) is a group of enzymes and peptides whose primary effector is Ang (angiotensin) II. The final effects of RAS activation depend not only on AngII synthesis through ACE (angiotensin-converting enzyme) but also on its degradation, which relies upon ACE2, a carboxypeptidase that degrades AngII to Ang-(1–7). Since Ang-(1–7) exerts opposing actions to those of AngII [1], ACE2 seems

Key words: angiotensin-(1–7), angiotensin-converting enzyme 2 (ACE2), atrial natriuretic peptide (ANP), diabetes, epithelial tubular cell, kidney disease.

Abbreviations: ACE, angiotensin-converting enzyme; ACE2KO, ACE2-knockout; ACEi, ACE inhibitor; Ang, angiotensin; ANP, atrial natriuretic peptide; ARB, AngII receptor blocker; AT, AngII type 1; AT, AngII type 2; BP, blood pressure; Coll, collagen 1; CTGF, connective tissue growth factor; CTL, control; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; iNOS, inducible NO synthase; MCP-1, monocyte chemotactic protein-1; NEP, neutral endopeptidase; NPR-A, natriuretic peptide receptor-A; PAS, periodate–Schiff; RAS, renin–angiotensin system; rACE2, recombinant ACE2; RT, reverse transcription.

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to counterbalance the circulating and vascular effects of AngII by both degrading bioactive AngII and producing bioactive Ang-(1–7) [1–3].

AngII is an important contributor to the pathogenesis of diabetic and non-diabetic kidney disease [4]. Both ACE and ACE2 are localized in the proximal tubules and the glomeruli where ACE2 may exert renoprotective effects [5]. Studies in ACE2-deficient mice have shown the progressive development of glomerulosclerosis [6]. Further works exploring the effects of ACE2 inhibition [7] or its genetic deletion [8] have demonstrated that ACE2 deficiency increases albuminuria as well as the associated glomerular lesions. In animal models [5] and human biopsies [9] of diabetic nephropathy, there is a reduction in ACE2 glomerular expression. Tikellis et al. [10] have also demonstrated that ACE2 is decreased in the diabetic tubule. Consistent with this, ACE2 overexpression has been reported to have a renoprotective effect since it prevents experimental diabetic glomerular injury [11]. However, the mechanisms for a reduction in ACE2 being associated with kidney disease, including the progression of diabetic nephropathy, have still to be fully clarified.

It has been recently shown that in the heart Ang-(1–7) increases ANP (atrial natriuretic peptide) [12]. ANP is an endogenous antagonist to AngII [13] both systemically and locally. In the kidney, this peptide has been shown to reduce oxidative stress [14] as well as to exert anti-proliferative [15], anti-fibrotic [16] and anti-inflammatory effects through the generation of cGMP. On the basis of these observations, we hypothesized that the kidney damage associated with ACE2 deficiency could partly depend on the loss of the renoprotective actions of ANP. Thus the aim of the present study was to evaluate whether and how ACE2 influences renal ANP.

**MATERIALS AND METHODS**

**Animals and experimental protocol**

**ACE2-deficient mice**

To evaluate the effect of ACE2 on renal ANP, two murine models of ACE2 deficiency were studied: 34 ACE2KO (ACE2-knockout) mice (on a C57BL/6 background) and streptozotocin-induced diabetes C57BL/6 mice. A total of 27 C57BL/6 [CTL (control)] and 27 ACE2KO male mice at 8 weeks of age were randomly allocated to three groups and were followed for 2, 12 and 22 weeks respectively. ACE2KO mice were provided by Professor Joseph Penninger (Institute of Molecular Biotechnology, Vienna, Austria) and were generated as described previously by his group [2,4]. At the same time, 54 male 8-week-old C57BL/6 mice were randomly allocated to receive streptozotocin (Sigma) at a dose of 55 mg/kg of body weight for the induction of diabetes (DM, n = 27) or buffer (sodium citrate buffer, pH 4.5; CTL, n = 27) in five consecutive daily doses. DM and CTL mice were then randomized further into three groups (n = 9 per group) and followed for 2, 12 and 22 weeks respectively. The mice were housed at the animal house of the Baker IDI Heart and Diabetes Institute of Melbourne and were studied according to NHMRC Institutional Guidelines for Care and Use of Laboratory Animals. The mice were fed a diet containing 0.2 % sodium by weight.

Of note, our group has demonstrated previously that the induction of diabetes in C57BL/6 mice following streptozotocin injections is associated with reduced renal ACE2 expression [17]. Specifically, both groups (ACE2KO and diabetic mice) demonstrated absent or reduced renal cortical ACE2 mRNA expression respectively (P < 0.001 compared with CTL) with reduced renal Ang-(1–7) concentrations in cortical homogenates (P < 0.05 compared with CTL) [17].

The mice that had been followed for 2 weeks, which were overall 10 weeks old, were put in metabolic cages for 24 h to collect their urine for biochemical analysis prior to killing. At the end of each study period, all the mice were killed after an intraperitoneal injection of Euthal (Delvet) at a dose of 10 mg/kg of body weight, and the kidneys were removed, weighed and snap frozen or put into formalin for further analysis.

**Ang-(1–7)-infused rats**

The effect of Ang-(1–7) on renal production of ANP was studied in 16 male Sprague–Dawley rats (body weight, 200–250 g) that were implanted with an osmotic minipump (model no. 2002; Alzet). For implantation of the minipumps, the animals were anaesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (Sigma) at a dose of 25 mg/100 mg of body weight. Buprenorfine (Temgesic, Reckitt Benckiser) was used as analgesic and was injected subcutaneously at a dose of 0.05 mg/kg of body weight on the day of the intervention and at a dose of 0.025 mg/kg of body weight on the day after. A total of eight rats were infused with Ang-(1–7) subcutaneously (24 µg/kg of body weight per h) and eight rats were infused with 0.9 % saline. After 10 days of study, rats were killed by an injection of 2,2,2-tribromoethanol at a dose of 25 mg/100 g of body weight and the kidneys were removed and snap frozen [18].

**Gene expression quantification by real-time PCR**

For the gene expression analysis, 3 µg of total RNA extracted from the kidneys of the 10-week-old mice was used to synthesize cDNA with Superscript First Strand synthesis system for RT (reverse transcription)–PCR, as described previously [19]. The renal gene expression
of ACE2, ANP, CTGF (connective tissue growth factor), Col (collagen) 1, Col4, fibronectin, ICAM-1 (intercellular adhesion molecule 1), iNOS (inducible NO synthase), IL-1β (interleukin-1β), IL-6 and MCP-1 (monocyte chemotactic protein-1) was analysed by real-time quantitative RT-PCR using the TaqMan system based on real-time detection of accumulated fluorescence.

Renal ANP gene expression was measured following the same method in RNA extracted from the kidneys of the Ang-(1–7)-infused rats and their controls.

### Renal morphology and ANP immunostaining
Paraffin sections (4 μm thick) were obtained from 4% (w/v) paraformaldehyde-fixed paraffin-embedded kidneys and were stained by nitrotyrosine immunostaining for nitrosylation of proteins by PAS (periodate–Schiff) for glomerulosclerosis and by Masson’s trichrome for renal fibrosis. To detect renal ANP both mouse and rat sections were stained with an anti-ANP antibody. In both immunostainings after neutralizing endogenous peroxidases, the sections of kidneys were incubated with rabbit anti-nitrotyrosine antibodies diluted 1:100 (Upstate) and with rabbit anti-ANP antibodies diluted 1:200 (Millipore). Biotinylated immunoglobulins (Vector Laboratories), diluted 1:500 and 1:200, were then applied with 3,3′-diaminobenzidine tetrahydrochloride (Sigma). Sections were then counterstained with haematoxylin and eosin. Each section was examined by light microscopy (Olympus BX50WI) and digitized with a high-resolution camera (Q-Imaging Fast 1394). The proportional areas of brown staining, bright pink staining and blue staining were then calculated to quantify the nitrosylation of proteins, ANP expression in the tubuli, glomerulosclerosis and fibrosis of the glomeruli respectively using an image analysis system (Image Pro Plus 6.3 Software; Media-Cybernetics).

### General parameters and biochemical data
Intrarenal concentrations of Ang-(1–7) were measured on snap-frozen renal cortical samples by a commercial RIA (ProSearch International), as described previously [17]. The measurement of urinary cGMP was performed using an EIA (enzyme immunoassay) kit (581021; Cayman Chemical), according to manufacturer’s instructions. Albuminuria was measured by ELISA (Bethyl Laboratories), according to manufacturer’s instructions. Creatinine clearance was estimated from serum and urine concentrations, as determined by HPLC.

### In vitro experiments
The gene expression of ANP was determined in NRK-52E cells (rat non-transformed proximal tubular epithelial cells), which express ANP according to the previous reports [20]. NRK-52E cells were treated with rACE2 (recombinant ACE2) (50 ng/ml), Ang-(1–7) (0.1; 0.5; 1; 5; 10; 25 μM; 2588; Auspep), an agonist of the Mas receptor [the Ang-(1–7) receptor] AVE-0991 (10 μM) or AngII (1 nM; 2078; Auspep) for 3 days. Untreated cells were used as controls. Blockade of AT1 (AngII type 1) and AT2 (AngII type 2) receptors, both combined and separately, was achieved by treating with the AT1 receptor blocker valsartan (1 μM; Novartis) and with the AT2 receptor antagonist PD123319 (1 μM; Sigma) for 30 min prior to the addition of AngII [18].

### Statistical analysis
Statistical analysis was performed using SAS. Results are expressed as means ± S.E.M. One-way ANOVA was performed to evaluate the differences in continuous variables among the groups. A P < 0.05 was considered statistically significant.

### RESULTS

#### Molecular pathological renal changes in ACE2 deficiency
Pathological kidney microscopic abnormalities were observed in 10-weeks-old ACE2KO mice. Their kidneys showed a significant induction of pro-oxidative, pro-inflammatory and pro-fibrotic mediators, such as iNOS, IL-6, IL-1β, ICAM-1, MCP-1, Col1, Col4, CTGF and fibronectin respectively (1.5–4.5-fold increased, P < 0.05 compared with CTL) (Figure 1A). This was associated with an increase in oxidative stress (P < 0.001 compared with CTL), evaluated as the amount of nitrosylated proteins in the glomeruli as well as glomerulosclerosis (P < 0.005) and glomerular fibrosis (P < 0.05 compared with CTL) (Figure 1B). In our study, these molecular renal changes were not associated with overt renal dysfunction, since 10-week-old ACE2KO mice did not show any changes in water intake, diuresis, albuminuria and creatinine clearance with respect to their CTLs. Albuminuria was 43 ± 1.3 μg/day in ACE2KO mice and 46 ± 1.1 μg/day in CTLs, whereas creatinine clearance was 0.32 ± 0.02 ml/min in ACE2KO mice and 0.26 ± 0.03 ml/min in CTLs. However, later changes cannot be excluded [6,21].

#### ACE2 deficiency and ANP
Lower intrarenal concentrations of Ang-(1–7) were found in 10-week-old ACE2KO mice (14.56 ± 0.81 fmol/mg in the CTL group and 5.98 ± 0.17 fmol/mg in the ACE2KO mice; P < 0.001). Genetic
ACE2 deficiency was also associated with a significant down-regulation of ANP gene expression in the kidneys of ACE2KO mice at 10, 20 and 30 weeks ($P < 0.05$ compared with CTL) (Figure 2A). This was confirmed by immunostaining of kidney sections in 10-week-old ACE2KO mice, which showed a significant decrease in tubular ANP ($P < 0.05$ compared with CTL) (Figures 2C–2E). The urinary levels of cGMP, which is the ANP second messenger, were also reduced in the ACE2KO mice, although not significantly. In the CTL mice at 10 weeks of age, urinary cGMP levels were $7398.5 \pm 820.1$ pmol/l, whereas they were $6650.2 \pm 741.8$ pmol/ml in ACE2KO mice.

The same findings were observed in a mouse model of acquired ACE2 deficiency, with 10-week-old diabetic mice having a reduction in renal ACE2 mRNA expression compared with the CTL group ($P < 0.01$). Accordingly, Ang-(1–7) levels were also significantly increased in diabetic mice being $2.09 \pm 0.12$ fmol/mg ($P < 0.001$ compared with CTL). As observed in ACE2KO mice, the acquired ACE2 deficiency of diabetic mice was associated with a significant down-regulation of ANP gene expression in the kidney at 10, 20 and 30 weeks ($P < 0.05$ compared with CTL) (Figure 2B). Moreover, the urinary levels of cGMP were also significantly reduced in diabetic mice ($3802.9 \pm 472.7$ pmol/ml; $P < 0.005$ compared with CTL).

In vitro and in vivo effects of Ang-(1–7) and other inhibitors and agonists of RAS signalling

Treatment of NRK-52E cells with Ang-(1–7) significantly up-regulated ANP gene expression in a dose-dependent manner until a plateau was reached (Figure 3A). The maximum response was observed when cells were treated with Ang-(1–7) at a dose range between 5 and 10 $\mu$M (Figure 3A). Consistent with this effect, exposing these cells to rACE2 (50 ng/ml) or the Mas receptor agonist AVE-0991 (10 $\mu$M) significantly increased ANP gene expression ($P < 0.05$ compared with CTL) (Figure 3B). In contrast, AngII did not have any significant effect on ANP gene expression (Figure 3C).

It has been shown that RAS blockade leads also to an increase in Ang-(1–7) levels by the degradation of an excess of AngII [2], therefore we treated our cells with the AT$_1$ receptor blocker valsartan (1 $\mu$M) and the AT$_2$ receptor blocker PD123319 (1 $\mu$M) in the presence or absence of AngII (1 nM). Tubular cells significantly up-regulated ANP gene expression when they were treated with both AT$_1$ and AT$_2$ receptors.
ACE2 and regulates ANP through Ang-(1–7)

**DISCUSSION**

The novel finding of the present study is that, in the kidney, unlike the heart where the synthesis of ANP is dependent on volume expansion and pressure overload, ACE2 directly regulates renal ANP production through the generation of Ang-(1–7). This is in agreement with the previous finding that Ang-(1–7) increases cardiac ANP secretion at high atrial pacing [12]. In the in vivo study, ACE2 deficiency (either genetic or acquired), which is characterized by low levels of Ang-(1–7) [17] and high levels of AngII [2], was associated with a significant down-regulation of renal ANP. NEP (neutral endopeptidase) is also an enzyme involved in the metabolism of Ang-(1–7) and ANP. Although the present study did not examine the role of NEP in the generation of Ang-(1–7) and the cleavage of ANP, the fact that ACE2KO had low levels of Ang-(1–7) in their kidneys suggests that NEP may not have a relevant role in the local generation of Ang-(1–7) in our model [22].

Thus in ACE2 deficiency ANP down-regulation could be caused either by a decrease in Ang-(1–7) or to an increase in AngII. To clarify which mechanism was responsible, we treated epithelial proximal tubular cells, which are a local renal source of ANP [20], with both Ang-(1–7) and AngII. This conclusively established that the ACE2 effect was related to Ang-(1–7). These results were then confirmed in vivo by demonstrating that Ang-(1–7) infusion significantly increased renal ANP gene and protein expression. Consistent with these observations, it has also been shown that basal cGMP production was significantly increased in kidney slices from Ang-(1–7)-treated mice compared with saline-infused animals [23]. Interestingly, we also observed that blocking both

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Figure 2  
ACE2 deficiency and ANP

(A) Renal mRNA expression of ANP in the kidneys of C57BL/6 (CTL) and ACE2KO mice at 10, 20 and 30 weeks of age. mRNA expression is reported as relative gene units; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. (B) Renal mRNA expression of ANP in the kidneys of C57BL/6 (CTL) and diabetic (DM) mice at 10, 20 and 30 weeks of age. mRNA expression is reported as relative gene units; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. (C) Tubular ANP staining is reported as a percentage of the stained area; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. (D and E) Representative images of ANP immunostained sections of kidneys of CTL mice and ACE2KO (original magnification ×25).
AT₁ and AT₂ receptors led to a significant up-regulation of ANP gene expression, which was even greater after the addition of AngII. We speculate that the addition of AngII caused a further increase in local unbound AngII, which is the substrate for Ang-(1–7), and therefore this led to an increase in local Ang-(1–7) production. On the contrary, when tubular cells were treated either with an AT₁ or an AT₂ receptor blocker there was no increase in ANP gene expression, possibly because in our model the partial blockade did not lead to a significant increase in unbound AngII as the complete blockade did. Experimental evidence has shown that RAS blockade leads to an increase in Ang-(1–7), which is an important contributor to the protective effects of ACEis (ACE inhibitors) or ARBs (AngII receptor blockers). In spontaneously hypertensive rats, the combination of the

Figure 3  Effects of various peptides of the RAS on ANP expression in tubular cells

(A) Effects of increasing doses of Ang-(1–7) mRNA expression of ANP in proximal tubular epithelial cells is reported as relative gene units; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL; §P < 0.001 compared with CTL. (B) Effects of ACE2, Ang-(1–7) and the Mas agonist. mRNA expression of ANP in proximal tubular epithelial cells is reported as relative gene units; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. (C) AngII blockade in presence and absence of AngII. mRNA expression of ANP in proximal tubular epithelial cells is reported as relative gene units; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. val, valsartan; PD, PD123319.

Figure 4  Effect of Ang-(1–7) infusion on ANP protein expression

(A) Tubular ANP staining is reported as the percentage stained area; results are expressed as means ± S.E.M. *P < 0.05 compared with CTL. (B, C) Representative images of ANP immunostained sections of kidneys of saline-infused (Control) and Ang-(1–7)-infused rats (original magnification ×25).
ACE2 and regulates ANP through Ang-(1–7)

ACEi lisinopril and the ARB losartan has been reported to increase plasma levels of Ang-(1–7), which was thought to contribute to the anti-hypertensive effect of these drugs [24]. Moreover, the ARB olmesartan increased the cardiac levels of Ang-(1–7) through the up-regulation of ACE2 [25] and, in the kidney, treatment with an ACEi promoted ACE2 activity, leading to an increase in Ang-(1–7) levels [26,27]. Thus we postulate that the up-regulation of ANP gene expression that we observed in vitro after blocking both AT1 and AT2 receptors could have been due to the conversion of AngII into Ang-(1–7). This is also consistent with the report that ACEi and ARB directly stimulate ANP secretion in vivo [28].

The concept that ACE2 increases renal ANP through Ang-(1–7) highlights a new mechanism whereby ACE2 opposes AngII. Overall, ACE2 is believed to counterbalance the circulating and local effects of AngII by degrading active AngII and simultaneously producing bioactive Ang-(1–7) [1–3]. In the present study, we show that ACE2 increases renal ANP and this might represent a third mechanism whereby ACE2 counterbalances AngII [1], since ANP is an endogenous antagonist to AngII. Systemic administration of ANP induces natriuresis, diuresis and a fall in BP (blood pressure) [13]. Recently, its chemical analogues have also been demonstrated to be effective in inhibiting angiotensin-dependent hypertension and vascular damage [29]. The diuretic and natriuretic properties of ANP are related to its ability to inhibit AngII-dependent sodium and water transport in the proximal tubule and water transport in the cortical collecting duct [30]. In addition, ANP inhibits aldosterone release and antagonizes the distal tubular actions of aldosterone by binding its receptor A [31]. It also inhibits vasopressin release and stimulates vascular dilation [32]. Because of these properties, it has been proposed that one of the negative feedback mechanisms compensating for RAS activation is indeed the AngII-dependent stimulation of ANP release from the atria of the heart through both direct, via AT1 receptor, and indirect actions, via atrial distension.

Beyond the systemic regulation of BP, ANP also has local 'protective' anti-proliferative and anti-fibrotic effects [16,33] in different organs, including the kidney. In particular, among the autocrine and/or paracrine actions [20] that ANP exerts in the kidney, this peptide has been shown to reduce oxidative stress [14] as well as to exert anti-proliferative [15], anti-fibrotic [16] and anti-inflammatory effects through the generation of membrane-bound cGMP. Of note, ANP generates cGMP through the activation of a membrane-bound guanylate cyclase, whereas the soluble form of this enzyme is stimulated by NO [34]; since NO production is notably impaired in diabetes [35], this could account for the difference that we observed between urinary cGMP in ACE2KO and diabetic mice. Experimental evidence demonstrates that ANP inhibits cell growth of vascular smooth muscle cells, fibroblasts and mesangial cells [15,31,36], where the ANP/NPR-A (natriuretic peptide receptor-A)/cGMP system would inhibit the proliferative effects of AngII and or ET-1 (endothelin-1) [37]. Moreover, overexpression of BNP (brain natriuretic peptide) in mice attenuated renal injury in a model of renal ablation, glomerulonephritis and diabetic nephropathy [38–40]. This suggests that ANP exerts a paracrine direct protective effect within the kidney and it may protect against the progression of renal disease. Given all these effects of ANP, the finding that ACE2 deficiency reduces renal ANP could explain why ACE2-deficient mice, including diabetic mice, develop spontaneous kidney abnormalities. Notably, it has been demonstrated that the deletion of ACE2 leads to the development of AngII-dependent glomerular injury, which can be prevented by treatment with the ARB irbesartan [6]. Similar to what was reported in the study by Oudit et al. [6], we found that by 10 weeks of age ACE2-deficient mice have a significant up-regulation of genes involved in the generation of oxidative stress and inflammatory processes, as well as in fibrogenesis. In addition, ACE2-deficient mice display increased glomerular nitrosylation of proteins, which is a marker of oxidative stress, glomerulosclerosis and glomerular fibrosis. Thus all of these abnormalities could result from a local increase in AngII and a local decrease in both Ang-(1–7) and ANP. This is consistent with the report by Nishikimi et al. [16] showing a link between decreased ANP and increased profibrotic gene expression in the kidneys, where exogenous ANP administration inhibited renal fibrosis. To what extent the decrease in either Ang-(1–7) or ANP may contribute separately to the kidney phenotype of ACE2KO mice is difficult to assess given that Ang-(1–7) seems to stimulate ANP production and that there seems to be also a synergistic effect between both peptides [12]. For example, Shah et al. [12] have demonstrated that the blockage of NPR-A in the heart diminished the anti-hypertrophic effect of Ang-(1–7). Interestingly, given the renoprotective paracrine effects of ANP [14–16,37], the finding that ACE2 increases ANP in vitro could also explain why overexpression of ACE2 in diabetic mice results in amelioration of their glomerular injury [11]. Thus we speculate that the benefit of reintroducing ACE2 in diabetic mice might partly be due to an expected increase in renal ANP levels.

In conclusion, the results of the present study demonstrate that ACE2 is very important for the renal production of ANP through the generation of Ang-(1–7). This is a third and a new mechanism whereby ACE2 may oppose AngII. Moreover, in ACE2-deficient states, such as diabetes, the loss of the renoprotective effects of ANP could worsen the effects of increased levels of AngII, contributing to the development and progression of kidney disease, including diabetic nephropathy.
AUTHOR CONTRIBUTION

Stella Bernardi designed and performed the experimental work, analysed the data, and wrote, edited and revised the paper prior to submission. Wendy Burns and Barbara Toffoli performed the experimental work and analysed the data. Raelene Pickering, Despina Tzorotes, Edward Grix, Martyno Sakoda and Elena Velkoska assisted in the experimental work. Louise Burrell designed the work. Colin Johnston and Merlin Thomas designed the work, analysed the data, and wrote, edited and revised the paper prior to submission. Christos Tikellis designed and performed the experimental work, and edited the paper prior to submission.

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