

ACE2 and Ang-(1-7) Confer Protection Against Development of Diabetic Retinopathy

Amrish Verma¹, Zhiying Shan², Bo Lei³, Lihui Yuan², Xuan Liu¹, Takahiko Nakagawa⁴, Maria B Grant⁵, Alfred S Lewin⁶, William W Hauswirth¹, Mohan K Raizada² and QiuHong Li¹

¹Department of Ophthalmology, University of Florida, Gainesville, Florida, USA; ²Department of Physiology and Functional Genomics, University of Florida, Gainesville, Florida, USA; ³The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, Chongqing Eye Institute, Chongqing, China; ⁴Division of Renal Disease and Hypertension, University of Colorado Denver, Aurora, Colorado, USA;

⁵Department of Pharmacology and Therapeutics, University of Florida, Gainesville, Florida, USA; ⁶Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida, USA

Despite evidence that hyperactivity of the vasodeleterious axis (ACE/angiotensin II (Ang II)/AT1 receptor) of the renin–angiotensin system (RAS) is associated with the pathogenesis of diabetic retinopathy (DR) use of the inhibitors of this axis has met with limited success in the control of this pathophysiology. We investigated the hypothesis that enhancing the local activity of the recently established protective axis of the RAS, ACE2/Ang-(1-7), using adeno-associated virus (AAV)-mediated gene delivery of ACE2 or Ang-(1-7) would confer protection against diabetes-induced retinopathy. Genes expressing ACE2 and Ang-(1-7) were cloned in AAV vector. The effects of ocular AAV-ACE2/Ang-(1-7) gene transfer on DR in diabetic eNOS^{-/-} mice and Sprague-Dawley (SD) rats were examined. Diabetes was associated with approximately tenfold and greater than threefold increases in the ratios of ACE/ACE2 and AT1R/Mas mRNA levels in the retina respectively. Intraocular administration of AAV-ACE2/Ang-(1-7) resulted in significant reduction in diabetes-induced retinal vascular leakage, acellular capillaries, infiltrating inflammatory cells and oxidative damage in both diabetic mice and rats. Our results demonstrate that DR is associated with impaired balance of retinal RAS. Increased expression of ACE2/Ang-(1-7) overcomes this imbalance and confers protection against DR. Thus, strategies enhancing the protective ACE2/Ang-(1-7) axis of RAS in the eye could serve as a novel therapeutic target for DR.

Received 4 October 2010; accepted 30 June 2011; published online 26 July 2011. doi:10.1038/mt.2011.155

INTRODUCTION

Diabetic retinopathy (DR) is the most common diabetic vascular complication, and despite recent advances in therapeutics and management, DR remains the leading cause of severe vision loss in people under the age of 60.¹ The renin–angiotensin system (RAS) plays a vital role in the cardiovascular system. Angiotensin II (Ang II), a peptide hormone of the RAS, has been known to regulate a variety of hemodynamic physiological responses, including fluid

homeostasis, renal function, and contraction of vascular smooth muscle. Furthermore, Ang II is known to mediate a multitude of other effects, such as the induction of reactive oxygen species, cytokines, growth factors, and collagen synthesis.^{2–5} In addition to circulating RAS, increasing evidence implicates the involvement of the local RAS in retinal vascular dysfunctions. Various components of RAS have been detected in the eye.^{6–11} Elevated levels of renin, prorenin, and Ang II have been found in patients with DR. In fact ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs) have been shown to improve diabetes-induced retinal vascular, neuronal, and glial dysfunction.^{12–16} Recent clinical studies have also clearly demonstrated beneficial effects of RAS inhibition in both type 1 and type 2 diabetic patients with retinopathy.^{17–21} Despite these positive outcomes, RAS blockers are not completely retinoprotective and retinopathy still progress to late stage. This could be attributed to the existence of local Ang II formation and that the RAS blockers are unable to cross the blood-retina barrier (BRB) in a concentration sufficient to influence the local RAS in the eye. In addition, increasing evidence suggests that Ang II can be generated via multiple pathways that may not be blocked by classic ACEi.^{22–25} Furthermore, additional components of RAS that contribute to end-organ damage, such as the receptor for renin and prorenin, have been recently identified.²⁶ Activation of prorenin/pro/renin receptor signaling pathway can initiate RAS cascade independent of Ang II.²⁶

Discovery of angiotensin-converting enzyme 2 (ACE2) has resulted in the establishment of a novel axis of the RAS involving ACE2/Ang-(1-7)/Mas.^{27,28} This vasoprotective axis counteracts the traditional proliferative, fibrotic, proinflammatory and hypertrophic effects of the ACE/Ang II/AT1R axis of the RAS.²⁷ The importance of the vasodeleterious axis of the RAS [ACE/Ang II/AT1R] in cardiovascular disease, as well as in diabetes and diabetic complications, is well established since ACEi and ARBs are leading therapeutic strategies.^{29–32} However, the impact of the vasoprotective axis of the RAS remains poorly understood.^{27,33–35} The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis.^{27,36–40} Indeed, ACE2/Ang-(1-7) activation is now considered to be a critical part of the beneficial actions of ACEi

Correspondence: QiuHong Li, Department of Ophthalmology, University of Florida, Gainesville, Florida 32610-0284, USA. E-mail: qli@ufl.edu

and ARB drugs.^{27,33} We hypothesized that an imbalance in the vasoprotective versus vasodeleterious axis of the RAS, particularly within the ocular tissue, would result in the development and progression of DR and that enhancing the protective axis of ACE2/Ang-(1-7) at the tissue level would directly counteract the effects of Ang II, regardless of its intracellular sources of formation.

In this study, we tested this hypothesis by examining the retinal RAS gene expression during the progression of diabetic retinopathy and evaluating the effects of increased expression of ACE2/Ang-(1-7) in the retina using adeno-associated virus (AAV)-mediated gene transfer. We showed that intravitreal administration of AAV vector expressing ACE2 or Ang1-7 peptide reduced diabetes-induced retinal pathophysiology in two rodent models.

RESULTS

Expression of the retinal RAS genes in the eNOS^{-/-} mouse retinas during the progression of diabetes

We have previously shown that diabetes induced by streptozotocin (STZ) treatment in eNOS^{-/-} mice results in more severe, accelerated retinopathy than diabetes in eNOS^{+/+} animals.⁴¹ Thus, our first objective was to compare retinal mRNA levels of the RAS genes in control and diabetic animals during the progression of diabetes. We observed three to tenfold increases in the mRNA levels of the vasodeleterious axis of the RAS (angiotensinogen, renin, pro-renin receptor, ACE and AT1 receptor subtypes) following STZ treatment (Figure 1). In contrast, there was ~30% reduction in ACE2 mRNA following an initial brief stimulatory response. As a result ACE/ACE2 mRNA ratio was increased by tenfold, while AT1R/Mas ratio was increased by threefold following 1 month of diabetes (Figure 1). The Mas mRNA level was slightly increased early in diabetes (Figure 1), followed by more than 50% decrease by 2 month after diabetes. The reason for this discrepancy between mRNA levels and protein activity of ACE2 is still unclear. It could be due to changes in post-transcriptional modifications, or protein degradation/stability. And further studies will be needed to elucidate the underlying mechanisms. Nevertheless, these observations were our first indication that DR is associated with a shifting in balance of the retinal RAS toward vasodeleterious axis.

Characterization of AAV vectors expressing ACE2 and Ang-(1-7)

AAV vector expressing the secreted form of human ACE2 was constructed under the control of the chick-β-actin (CBA) promoter (Figure 2a). This secreted form of ACE2 has been previously characterized and shown to be enzymatically active.⁴² Since Ang-(1-7) peptide contains only 7 amino acids and small peptides are usually difficult to express in mammalian cells, we have designed an expression construct in which the Ang-(1-7) peptide is expressed as part of the secreted fusion green fluorescent protein (GFP) protein, which is subsequently cleaved upon secretion. The expression of the fusion sGFP-FC-Ang-(1-7) is under the control of the CBA promoter in the AAV vector (Figure 2a) and was confirmed by transfecting the HEK293 cells using this plasmid DNA (Figure 2b). To ensure that the fusion protein was indeed secreted, proteins isolated from the culture supernatants as well as cell lysates from transfected, sham-transfected or untransfected cells were analyzed by western blotting (Figure 2b).

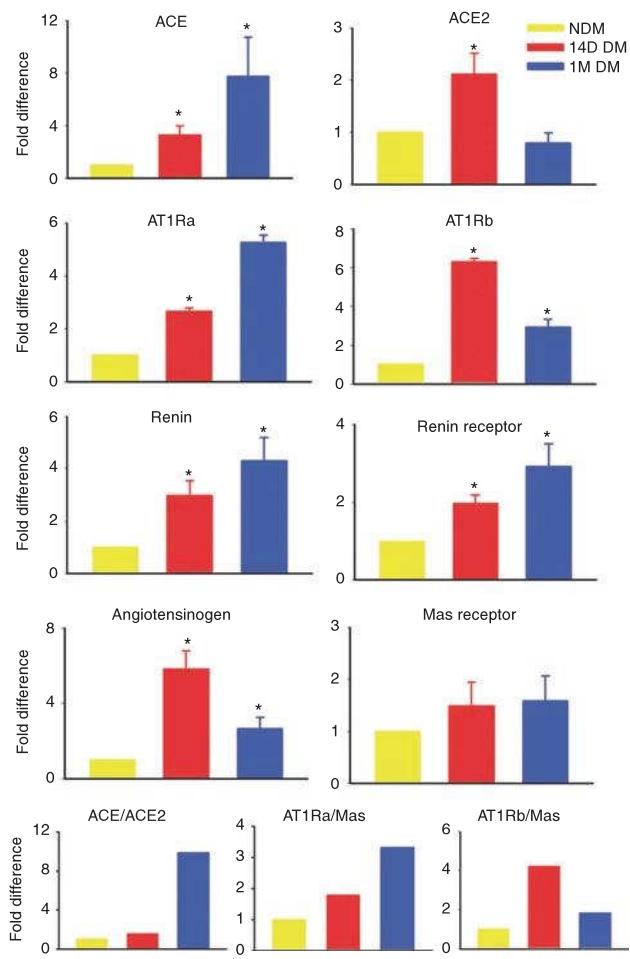


Figure 1 Real-time reverse transcriptase (RT)-PCR analysis of retinal mRNA for renin-angiotensin system genes. Values on y-axis represent fold difference compared to age-matched nondiabetic retinal samples for each gene at each time point (14 day and 1 month after induced diabetes). DM, diabetic; NDM, nondiabetic. At least four eyes were analyzed at each time point. * $P < 0.01$ (versus NDM group).

Mass spectrometry analysis of Ang (1-7) peptide in supernatant samples of HEK293 cells transfected with the sGFP-FC-Ang-(1-7) plasmid DNA was also performed. The Ang-(1-7) peptide was detectable in supernatant isolated from cells transfected with sGFP-FC-Ang-(1-7) plasmid DNA, but was not detectable in samples isolated from un-transfected cells, or cells transfected with the control plasmid expressing the cytoplasmic GFP (data not shown).

Intravitreal administration of AAV-Ang-(1-7) resulted in a robust transduction of retinal cells primarily inner retinal layer (Figure 2c-f). This was associated with an increase in both cellular and secreted Ang-(1-7) (Figure 2g-h). Similarly, ACE2 protein level was increased in the retina following transduction with AAV-ACE2 (Figure 2g).

Ocular gene delivery of ACE2/Ang-(1-7) via the AAV vector in the retina results increased ACE2 activities and Ang-(1-7) peptide levels

Induction of diabetes resulted in more than fivefold increase in ACE activities in the retinas of eNOS^{-/-} mice, whereas ACE2

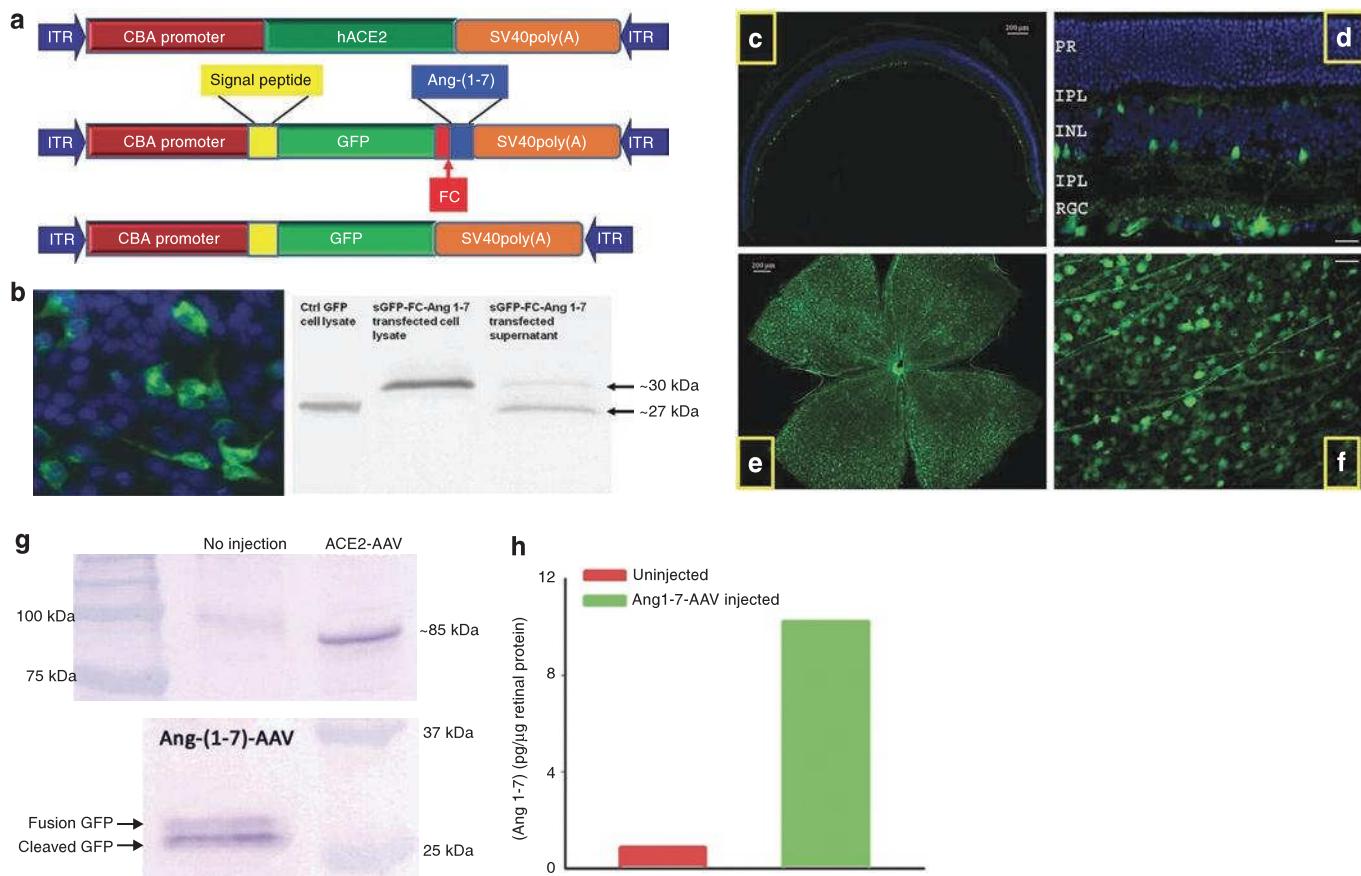


Figure 2 Construction and characterization of adeno-associated virus (AAV) vectors expressing angiotensin-converting enzyme 2 (ACE2) and Ang-(1-7). (a) Maps of the AAV vector expressing the human ACE2 gene (hACE2) and the AAV vector expressing Ang-(1-7) gene. The Ang-(1-7) peptide is expressed as part of fusion protein, and cleaved upon secretion at the furin cleavage (FC) site. CBA, CMV-chicken-β-actin promoter; ITR, inverted terminal repeat. A control vector contains coding region for the secreted green fluorescent protein (GFP) without Ang-(1-7) peptide coding sequence. **(b)** Expression and cleavage of the fusion protein. In cultured HEK293 cells transfected with the plasmid sGFP-FC-Ang-(1-7), or infected with AAV-sGFP-FC-Ang-(1-7), there was robust expression of GFP as expected. Proteins isolated from cell lysates contained a single protein band with molecular weight ~30 kDa, as predicted for the precursor (fusion protein), but culture supernatants contained two protein bands (30 kDa and a 27 kDa), indicating that the secreted protein is cleaved at the furin cleavage site as predicted. **(c-f)** Transduction of mouse retina with AAV vector expressing sGFP-FC-Ang-(1-7) and hACE2. A single intravitreal injection of 1 μl AAV vector (10⁹ vg/eye) resulted in efficient transduction of inner retinal cells, primarily retinal ganglion cells. **(c)** Low magnification of cross section of a mouse eye that received AAV2-sGFP-FC-Ang-(1-7) injection. **(d)** Higher magnification of the same eye. **(e)** A retinal whole mount showing GFP expression. **(f)** Higher magnification of the same retinal whole mount. Bar = 20 μm in **d** and **f**. **(g)** Western blot of proteins isolated from an uninjected eye and an eye injected with AAV2-ACE2 (top) and AAV2-sGFP-FC-Ang-(1-7) (bottom) compared to a molecular weight standard (right lane). **(h)** Ang-(1-7) peptide levels in the retina with and without AAV-sGFP-FC-Ang-(1-7) injection. There was more than a tenfold increase in Ang-(1-7) peptide level detected by using an Ang-(1-7) specific EIA kit (Bachem, San Carlos, CA) in retinas receiving injection of AAV-sGFP-FC Ang-(1-7). INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PR, photoreceptor; RGC, retinal ganglion cells.

activity was relatively unchanged (**Figure 3a**). AAV2-ACE2 injected retinas showed more than twofold increase in ACE2 enzymatic activities (**Figure 3a**) and this was associated with a reduced level of Ang II and increased Ang-(1-7) peptide level (**Figure 3b**), but had marginal effect on ACE activity (**Figure 3a**). Injection of AAV2-Ang-(1-7) had no effect on ACE2 activity, but significantly decreased ACE activity (**Figure 3a**).

We also determined Ang II and Ang-(1-7) peptide levels. STZ induced diabetes resulted in more than twofold increase in Ang II levels whereas Ang-(1-7) level was un-changed in the retinas of eNOS^{-/-} mice (**Figure 3b**). The increase of Ang II was completely normalized in retinas injected with AAV-ACE2 but was unchanged in retinas injected with AAV-Ang-(1-7) vector (**Figure 3b**).

Local ocular treatment with either AAV-ACE2 or AAV-Ang-(1-7) vector had no effects on body weight, blood glucose and blood pressure in diabetic eNOS^{-/-} mice (**Supplementary Table S2**).

Increased ACE2/Ang1-7 expression in the retina reduced diabetes-induced retinal pathophysiology

We investigated if elevated retinal expression of ACE2 or Ang-(1-7) would overcome vasodeleterious effect of ACE/AT1R axis and prevent the development of diabetes-induced retinopathy. The effect of increased ACE2 and Ang-(1-7) expression on retinal vascular permeability was evaluated by fluorescein isothiocyanate-labeled albumin extravasations and quantified by measuring fluorescent intensity in serial sections from nondiabetic, untreated, ACE2

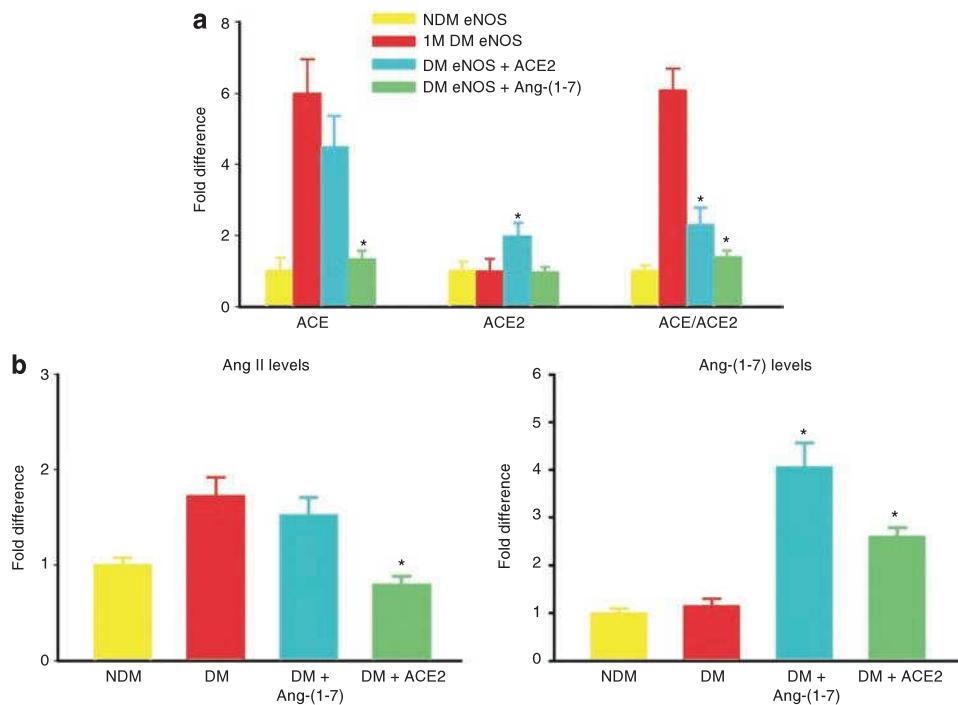


Figure 3 Angiotensin-converting enzyme (ACE), ACE2 activities, and angiotensin peptide levels in the retinas. **(a)** ACE and ACE2 enzymatic activities and ACE/ACE2 ratios in nondiabetic (NDM), 1 month diabetic (1M DM), and 1 month diabetic eNOS^{-/-} retinas treated with AAV-ACE2/Ang-(1-7). Values are expressed as fold differences compared with age-matched nondiabetic group. *P < 0.01 (versus untreated DM group, N = 6/group). **(b)** Ang II and Ang-(1-7) peptide levels in nondiabetic (NDM), 1 month diabetic (1M DM), and 1 month diabetic eNOS^{-/-} retinas treated with AAV-ACE2/Ang-(1-7), measured by ELISA using a commercial kit. *P < 0.01 (versus untreated DM group). Values represent fold difference compared with age-matched nondiabetic group. Three retinas were pooled for each measurement, each measurement was done in duplicates, and three separate pools were averaged for each group.

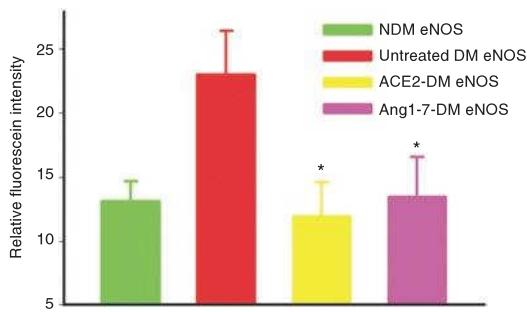


Figure 4 Effects of ocular treatments with angiotensin-converting enzyme (ACE2) and Ang-(1-7)-AAV2 on retinal vascular permeability in diabetic eNOS^{-/-} mice. Retinal vascular permeability was evaluated by fluorescein isothiocyanate (FITC)-labeled albumin extravasations and quantified by measuring the fluorescent intensity in serial sections from eNOS^{-/-} mice at 1 month after induced diabetes. Data are presented as mean ± SD from six eyes in each group. *P < 0.01 (versus untreated DM group). DM, diabetes; NDM, nondiabetes.

and Ang 1-7 treated diabetic eNOS^{-/-} mice. Induction of diabetes for 2 month in eNOS^{-/-} mice resulted in twofold increase in vascular permeability, this pathophysiology was significantly reduced in diabetic retinas that received ACE2/Ang-(1-7) vector treatments (**Figure 4**), but not in the retinas that received treatment with control vector which contained the coding sequence for secreted GFP without Ang-(1-7) or ACE2 (data not shown). Diabetes induced increased infiltrating CD45 positive macrophages and activation of CD11b positive microglial cells; this

was significantly reduced in eyes treated with ACE2 and Ang-(1-7) expression vectors (**Figure 5**).

Induction of diabetes for 2 months in eNOS^{-/-} mice resulted in greater than tenfold increase in the formation of acellular capillaries and this was significantly reduced in diabetic retinas which received ACE2/Ang-(1-7) vector treatments (**Figure 6**). Furthermore, this increase in the level of ACE2 also prevented the basement membrane thickening in diabetic eNOS^{-/-} retina (**Supplementary Figure S1**).

Finally, we used STZ-induced diabetes in SD rats as an additional animal model of diabetes to provide further conceptual validation. We observed more than fivefold increase in the number of acellular capillaries in STZ-induced diabetic rat retinas at 14 month of diabetes. This increase was almost completely prevented by gene delivery of either ACE2 or Ang-(1-7) (**Figure 7**).

Again ocular treatment with either AAV-ACE2 or AAV-Ang-(1-7) vector had no effects on body weight, blood glucose and blood pressure in diabetic SD rats (**Supplementary Table S3**).

Increased expression of ACE2/Ang-(1-7) reduced oxidative damage in diabetic retina

Diabetes and its complications are associated with increased oxidative stress. We assessed oxidative damage measuring the levels of thiobarbituric acid-reactive substances (a marker for oxidative damage)⁴³ in the retina. Diabetes induced a significant increase in thiobarbituric acid-reactive substances (**Figure 8a**) in

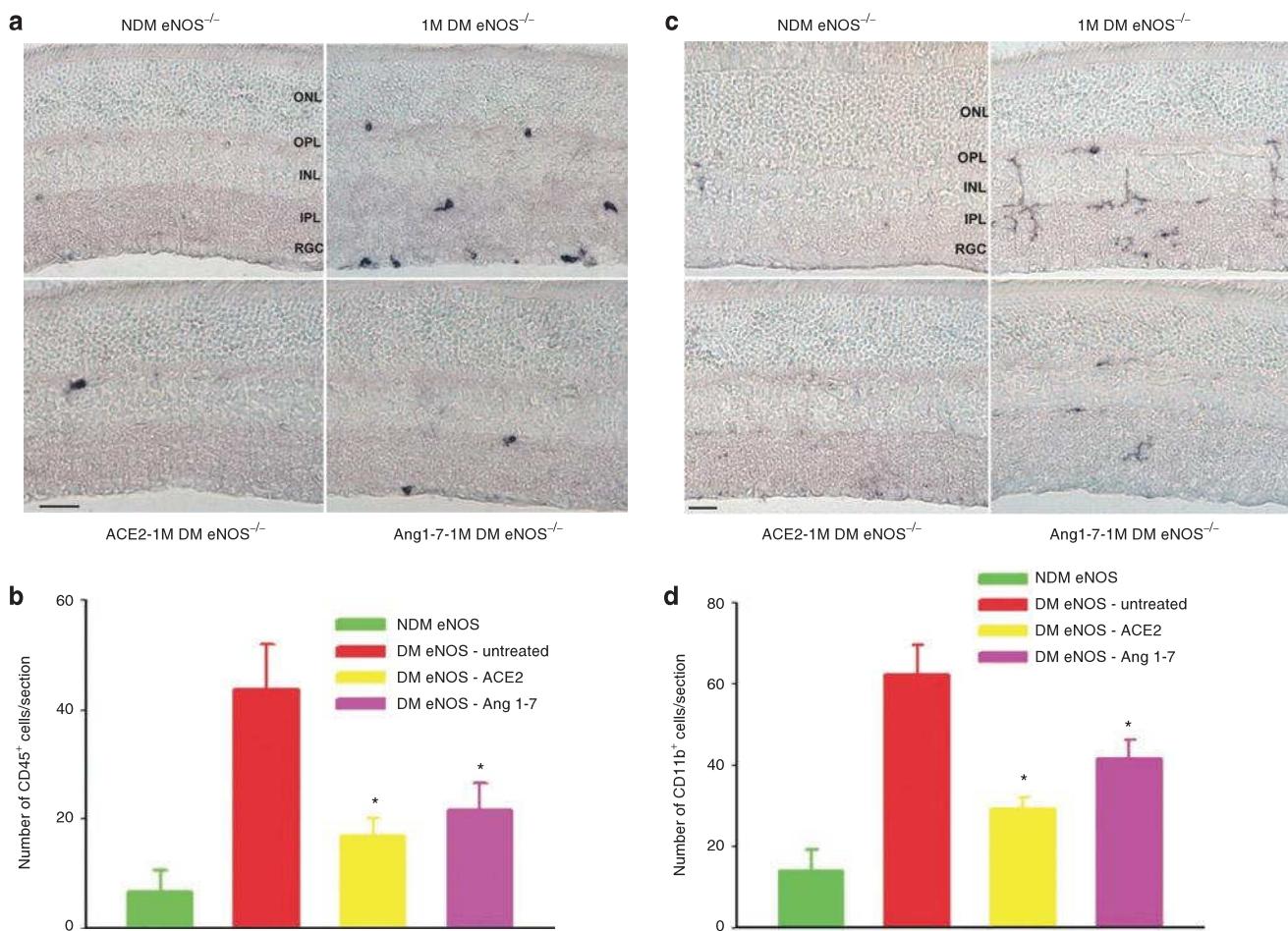


Figure 5 Intravitreal administration of ACE2 or Ang-(1-7)-AAV reduces diabetes-induced ocular inflammation. **(a)** Representative images of CD45⁺ cells in the nondiabetic, untreated, ACE2, and Ang-(1-7)-treated diabetic eNOS^{-/-} mouse retinas at 1 month after induced diabetes. **(b)** Quantification of CD45⁺ inflammatory cells in the retinas from the nondiabetic, untreated, ACE2, and Ang-(1-7)-treated diabetic eNOS^{-/-} mouse retinas at 1 month after induced diabetes. **(c)** Representative images of CD11b⁺ cells in the nondiabetic, untreated, ACE2, and Ang-(1-7)-treated diabetic eNOS mouse retinas at 1 month after induced diabetes. **(d)** Quantification of CD11b⁺ inflammatory cells in the retinas from the nondiabetic, untreated, ACE2, and Ang-(1-7)-treated diabetic eNOS mouse retinas at 1 month after induced diabetes. $N = 4$ for each group. * $P < 0.01$ (versus untreated DM group). Bar = 50 μ m. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cells.

eNOS^{-/-} mouse retinas (Figure 8a). This increase was completely prevented by AAV-ACE2 or Ang-(1-7) treatment. Similar results were also obtained in SD rat retinas (Figure 8b).

DISCUSSION

We demonstrate that all the genes within the RAS are expressed in the retina, consistent with various previous reports (reviewed in ref. 10 and references therein), and that the expression levels of genes in the vasoconstrictive arm of RAS (renin, ACE, AT1R) are highly elevated in diabetic retinas. In contrast, there is an initial increase in the expression of genes in the vasoprotective axis (ACE2 and MAS) early in diabetes; this is reduced during the diabetes progression, thus tipping the balance toward more vasoconstrictive, proinflammatory, hypertrophic effects of RAS mediated by ACE/Ang II/AT1R axis. This imbalance of local RAS is associated with increased ACE activity and Ang II levels in the diabetic retinas and unchanged ACE2 activity and Ang-(1-7), although the ACE2 and Mas receptor mRNA levels are reduced.

Furthermore, we demonstrate that enhanced expression of either ACE2 or Ang-(1-7) via AAV vector mediated gene delivery in the retina prevents diabetes-induced retinal vascular permeability, thickening of basement membrane, retinal inflammation, formation of acellular capillaries, and oxidative damage in both mouse and rat models of diabetic retinopathy. More importantly, these beneficial effects occur in the absence of systemic improvement of glucose, blood pressure, both of which are elevated in eNOS^{-/-} mice⁴¹ (Supplementary Table S2), and other diabetic complications,⁴⁴ suggesting that dysregulation of local RAS plays a significant role in the pathogenesis of diabetic retinopathy, and can be modulated locally to restore the balance between the two counter-acting arms by enhancing the ACE2/Ang-(1-7)/MAS axis. These observations provide conceptual support that enhancing ACE2/Ang-(1-7) axis maybe an effective strategy for the treatment of DR.

Although various components of RAS have been detected in retina, our study is the first to examine the expression levels

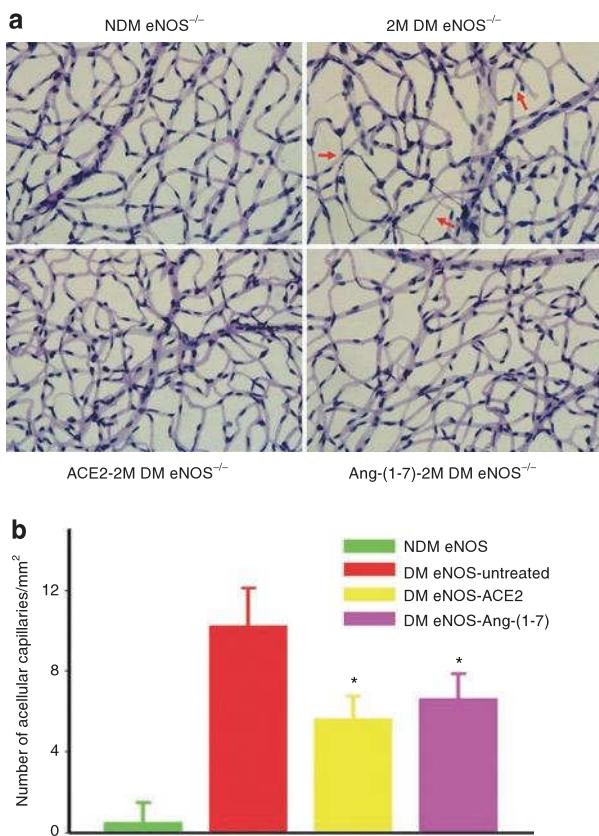


Figure 6 Evaluation of acellular capillary formation in untreated and AAV-ACE2/Ang-(1-7) treated retinas of diabetic mice. Treatments with ACE2 and Ang 1-7 vectors in the diabetic eNOS^{-/-} mouse retinas reduced acellular capillaries. **(a)** Representative images of trypsin-digested retinal vascular preparations from nondiabetic eNOS^{-/-}, untreated, ACE2, and Ang-(1-7)-treated diabetic eNOS^{-/-} mouse retinas (2 month after induced diabetes). Arrows indicate the acellular capillaries. **(b)** Quantitative measurements of acellular capillaries. The values on y-axis represent the number of acellular capillaries/mm² retina. DM, diabetes; NDM, nondiabetes. N = 6. *P < 0.01 (versus untreated DM group).

of all known RAS genes during the progression of diabetes in the eNOS^{-/-} mice, which exhibit accelerated retinopathy.⁴¹ We show that increased expression of genes in the vasoconstrictive, proinflammatory axis of RAS (ACE, AT1R, renin, renin receptor) occur early, 14 days after STZ-induced diabetes. We have previously shown that increased retinal vascular permeability and gliosis are already detectable at this time point in diabetic eNOS^{-/-} mouse retina, suggesting that local hyperactivity of the deleterious axis (ACE/Ang II/AT1R) may contribute to these pathological changes. We also measured ACE and ACE2 activities in diabetic eNOS^{-/-} mouse retina. In contrast to a previous report which showed that ACE enzyme activity was decreased, and ACE2 enzyme activity was increased in diabetic rat retinas,¹¹ we found that ACE activity is highly increased in these diabetic retinas, whereas ACE2 activity remained unchanged. This discrepancy may be due to the difference in animal models or the time points examined.

The importance of the vasodeleterious axis of the RAS (ACE/ Ang II/ AT1R) in cardiovascular disease, as well as in diabetes and

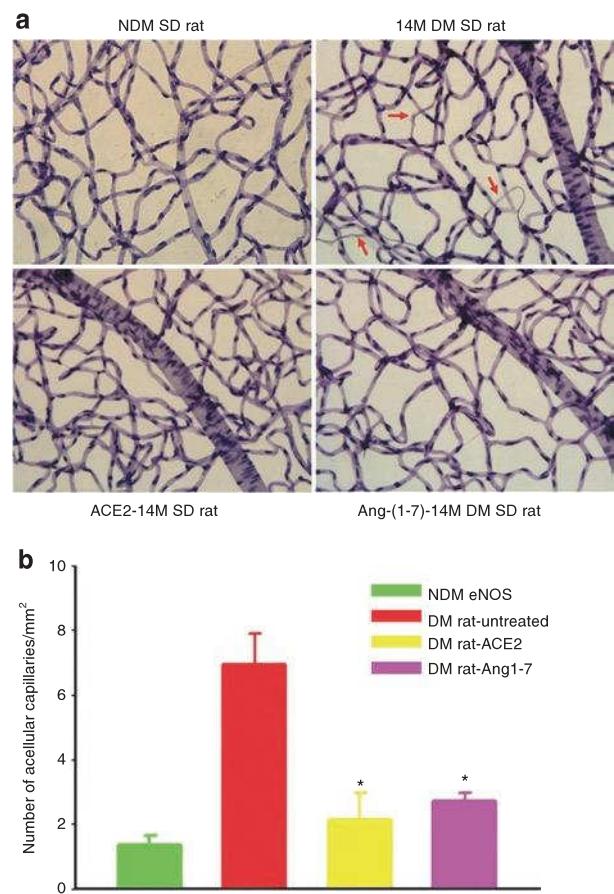


Figure 7 Evaluation of acellular capillary formation in untreated and ACE2/Ang-(1-7) AAV2 vectors treated retinas of diabetic SD rats. **(a)** Representative images of trypsin-digested retinal vascular preparations from nondiabetic SD rat, untreated, ACE2, and Ang-(1-7)-treated diabetic SD rat retinas (14 month after induced diabetes). **(b)** Quantitative measurements of acellular capillaries. The values on y-axis represent the number of acellular capillaries/mm² retina. DM, diabetes; NDM, nondiabetes. N = 6. *P < 0.01 (versus untreated DM group).

diabetic complications, is well established since ACEi and ARBs are leading therapeutic strategies.^{29–32} However, the impact of the vasoprotective axis of the RAS remains poorly understood, particularly in the eye. The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis.^{27,33–35} In agreement with these studies, we now show that increased expression of either ACE2 or Ang-(1-7) is protective in both diabetic eNOS^{-/-} mouse and rat model of diabetic retinopathy. The protective effect of ACE2 appears to be a result of reduced Ang II, by catalyzing its conversion to Ang-(1-7), thus increasing the level of Ang-(1-7), or a combination of both. Indeed, in the AAV-ACE2 injected retina, the diabetes-induced elevation of Ang II was reduced and this was associated with increased level of Ang-(1-7). However, the fact that increased Ang-(1-7) expressed from AAV vector in the retina was equally protective despite high Ang II levels suggests that Ang-(1-7) alone can directly counteract the effect of high retinal Ang II levels in diabetes. This is consistent with well-established effects of Ang-(1-7).⁴³

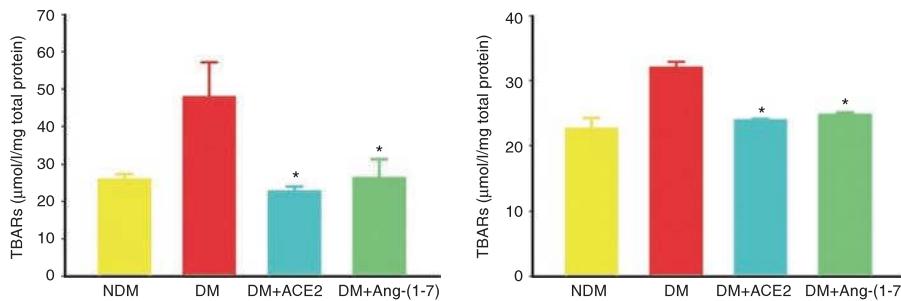


Figure 8 Thiobarbituric acid-reactive substances (TBARs) levels in *eNOS*^{-/-} mouse retinas (left) and SD rat retinas (right). Diabetes resulted in increased TBARs levels in both *eNOS*^{-/-} mouse retinas (1 month diabetes) and SD rat retinas (at 4 month diabetes). These increases were prevented by AAV-ACE2/Ang-(1-7) treatments. DM, diabetes; NDM, nondiabetes. $N = 6/\text{group}$. * $P < 0.01$ (versus untreated DM).

It is interesting to note that ACE2 overexpression was associated with reduced Ang II and increased Ang-(1-7) levels as expected, but had no effect on ACE activity. In contrast, overexpression of Ang-(1-7) had no effect on endogenous ACE2 activity, but significantly reduced ACE activity. Paradoxically, despite reduced ACE activity in AAV-Ang-(1-7) injected retina, Ang II level remained high. It is possible that other enzymes/pathways besides ACE maybe involved in the formation of Ang II. One such candidate is the chymase, which has been detected in vascular system and other tissues including eye.⁴⁶ Another candidate pathway is the receptor for prorenin and renin (pro/renin). Binding of pro/renin to its receptor, pro/renin receptor, causes its prosegment to unfold, thereby activating prorenin so that it is able to generate angiotensin peptides that stimulate the Ang II-dependent pathway.²⁶ Considering the fact that retina contains a high level of prorenin, which is further increased in patients with diabetic retinopathy,⁶ this pathway may contribute to the increased Ang II level that is observed in diabetes. The existence of multiple pathways for Ang II formation at the tissue level may explain the limited beneficial effects of classic RAS blockers, and also lend the support for the notion that enhancing the protective axis of RAS (ACE2/Ang-(1-7)/Mas) may represent a more effective strategy for treatment of diabetic retinopathy and other diabetic complications.

AAV vector mediated gene therapy for ocular diseases has been studied in animal models for more than a decade. Reports focusing on retinal therapy include a wide variety of retinal degenerative animal models of corresponding human retinopathies, as well as the therapeutic effects of AAV-vector mediated expression of neuroprotective, anti-apoptotic, anti-angiogenic agents in the retina.⁴⁷ In view of recent clinical trials,⁴⁸ gene therapy has emerged as viable approach and may become treatment options for a range of diseases in the future. In particular, when considering that a diabetic individual experiences ocular complications for decades, a therapeutic strategy that is long-lasting and that does not require repeated and frequent administration is desirable. Thus, the delivery of ACE2 and/or Ang-(1-7) could serve as a novel gene therapy target for DR in combination with existing strategies to control hyperglycemia and hypertension.

In summary, our observations demonstrate that diabetes-induced retinopathy is associated with an imbalance in the local RAS as a result of activation of the deleterious axis (ACE/Ang II/AT1R) and decrease in the vasoprotective axis (ACE2/Ang-1-7/Mas) of the RAS. Restoring this balance by ACE2 or Ang-(1-7)

overexpression provides profound protective effects against this pathophysiology. Thus, these results provide “proof-of-principle” that enhancing ACE2/Ang-(1-7)/MAS axis may represent a promising approach for treating DR and other diabetic complications.

MATERIALS AND METHODS

Animals and Experimental procedures. All the animal procedures adhere to protocols approved by the University of Florida Institutional Animal Care and Use Committee. Two animal models of DR were studied: the STZ-induced diabetic *eNOS*^{-/-} mouse model, which has been recently shown to develop accelerated retinopathy,⁴¹ and the STZ-induced diabetic SD rat model.

Breeding pairs of *eNOS*^{-/-} mice were purchased from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME) and male SD rats (8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). All animals were maintained at the Animal Care Service at the University of Florida. All procedures adhere to ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and approved protocols by the Animal Care and Use Committee of the University of Florida. Animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12–12-hour light–dark cycle.

Diabetes was induced by a single intraperitoneal injection of freshly prepared STZ solution (65 mg/kg body weight in 10 mmol/l citrate buffer, pH 4.5) for rats. Two consecutive intraperitoneal injections of STZ (100 mg/kg body weight, freshly made in 0.05 mol/l citrate buffer, pH 4.5) were used to induce diabetes in mice (male, ~8 weeks old). Diabetes was confirmed one week after STZ treatment by measuring the blood glucose level (defined >200 mg/dl) using the FreeStyle glucomonitor and test strip according to the manufacturer’s instruction.

Measurements of mRNA levels of retinal renin-angiotensin genes and inflammatory cytokines. Total RNA was isolated from freshly dissected retinas using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. Reverse transcription was performed using Enhanced Avian HS RT-PCR kit (Sigma, St Louis, MO) following manufacturer’s instructions. Real time PCR was carried out on a real time thermal cycler (iCycler, Bio-Rad Life Sciences) using iQTM Syber Green Supermix (Bio-Rad Life Sciences, Hercules, CA). The threshold cycle number (C_t) for real-time PCR was set by the cycler software. Optimal primer concentration for PCR was determined separately for each primer pair. Each reaction was run in duplicate or in triplicate, and reaction tubes with target primers and those with GAPDH/Actin primers were always included in the same PCR run. To test the primer efficiencies, the one-step reverse transcriptase-PCR was run with each target primer. Relative quantification was achieved by the comparative $2^{-\Delta\Delta C_t}$ method 1. The relative increase/decrease (fold-induction/repression) of mRNA of target X in the experimental group (EG) was calculated using the control group as the

calibrator: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is $\{Ct.x [EG] - Ct. GAPDH [EG]\} - \{Ct.x [control] - Ct. GAPDH [control]\}$. Primer sequences used in this study are shown in **Supplementary Table S1**.

AAV vectors and other procedures. An AAV vector containing a secreted form of human ACE2 gene under the control of the chimeric CMV/chicken β-actin promoter was constructed. We previously showed that the secreted ACE2 protein expressed from a lentiviral vector is enzymatically active.⁴² The construction and characterization of Ang-(1-7) peptide expression vector is described in **Supplementary Data**. To determine the protective role of ACE2 and Ang-(1-7), the vectors were administered by intravitreal injection two weeks before STZ induction of diabetes. Only one of eyes for each animal was injected and the contralateral eye was used as a control. General parameters (body weight, glucose levels, and blood pressure) of these animals with and without treatments are shown in **Supplementary Table S2** for mice and **Supplementary Table S3** for rats in **Supplementary Materials and Methods**.

Retinal vascular permeability was evaluated at 1 month after induction of diabetes, the loss of retinal capillaries was evaluated at 2 months after induction of diabetes for eNOS^{-/-} mice, and 14 month after induction of diabetes for SD rats. These time points were chosen based on the previously characterization of retinal vascular changes.⁴¹ At least six animals were used for each type of end-point assays.

Methods for retinal vascular evaluation (permeability, acellular capillaries, immunohisto-chemistry analysis) are essentially the same as described previously,⁴¹ and are presented in **Supplementary Data**.

Statistical analysis. All values are presented as mean ± SD. Paired Student's *t*-test was used to assess significance between two groups. One-way analysis of variance followed by the *post hoc* Tukey (Fisher's protected least significant difference) test was used to assess statistical significance between multiple groups. Differences were considered significant at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. Transmission electron micrographs of retinal capillaries of diabetic retina with and without vector treatment.

Table S1. Primers used for real-time RT-PCR analysis.

Table S2. General parameters of eNOS^{-/-} mice.

Table S3. General parameters of SD rats.

Materials and Methods.

Supplementary Data.

ACKNOWLEDGMENTS

This work was supported by a grant from American Diabetes Association, NIH grants EY11123, HL33610 and HL56921, Foundation Fighting Blindness, Juvenile Diabetes Research Foundation, and Research to Prevent Blindness, Inc. W.W.H. is the co-founder of AGTC Inc. A patent application for Ang-(1-7) expression vector by University of Florida is pending. The other authors declared no conflict of interest.

REFERENCES

- Cheung, N, Mitchell, P and Wong, TY (2010). Diabetic retinopathy. *Lancet* **376**: 124–136.
- Mehta, PK and Griendling, KK (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol, Cell Physiol* **292**: C82–C97.
- Marchesi, C, Paradis, P and Schiffrin, EL (2008). Role of the renin-angiotensin system in vascular inflammation. *Trends Pharmacol Sci* **29**: 367–374.
- de Cavanagh, EM, Inserra, F, Ferder, M and Ferder, L (2007). From mitochondria to disease: role of the renin-angiotensin system. *Am J Nephrol* **27**: 545–553.
- de Cavanagh, EM, Ferder, M, Inserra, F and Ferder, L (2009). Angiotensin II, mitochondria, cytoskeletal, and extracellular matrix connections: an integrating viewpoint. *Am J Physiol Heart Circ Physiol* **296**: H550–H558.
- Danser, AH, van den Dorpel, MA, Deinum, J, Derkx, FH, Franken, AA, Peperkamp, E et al. (1989). Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J Clin Endocrinol Metab* **68**: 160–167.
- Danser, AH, Derkx, FH, Admiraal, PJ, Deinum, J, de Jong, PT and Schalekamp, MA (1994). Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci* **35**: 1008–1018.
- Deinum, J, Derkx, FH, Danser, AH and Schalekamp, MA (1990). Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology* **126**: 1673–1682.
- Paul, M, Poyan Mehr, A and Kreutz, R (2006). Physiology of local renin-angiotensin systems. *Physiol Rev* **86**: 747–803.
- Fletcher, EL, Phipps, JA, Ward, MM, Vessey, KA and Wilkinson-Berka, JL (2010). The renin-angiotensin system in retinal health and disease: Its influence on neurons, glia and the vasculature. *Prog Retin Eye Res* **29**: 284–311.
- Tikellis, C, Johnston, CI, Forbes, JM, Burns, WC, Thomas, MC, Lew, RA et al. (2004). Identification of angiotensin converting enzyme 2 in the rodent retina. *Curr Eye Res* **29**: 419–427.
- Zhang, JZ, Gao, L, Widness, M, Xi, X and Kern, TS (2003). Captopril inhibits glucose accumulation in retinal cells in diabetes. *Invest Ophthalmol Vis Sci* **44**: 4001–4005.
- Zhang, JZ, Xi, X, Gao, L and Kern, TS (2007). Captopril inhibits capillary degeneration in the early stages of diabetic retinopathy. *Curr Eye Res* **32**: 883–889.
- Moravski, CJ, Skinner, SL, Stubbs, AJ, Sarlos, S, Kelly, DJ, Cooper, ME et al. (2003). The renin-angiotensin system influences ocular endothelial cell proliferation in diabetes: transgenic and interventional studies. *Am J Pathol* **162**: 151–160.
- Phipps, JA, Wilkinson-Berka, JL and Fletcher, EL (2007). Retinal dysfunction in diabetic ren-2 rats is ameliorated by treatment with valsartan but not atenolol. *Invest Ophthalmol Vis Sci* **48**: 927–934.
- Wilkinson-Berka, JL, Tan, G, Jaworski, K and Ninkovic, S (2007). Valsartan but not atenolol improves vascular pathology in diabetic Ren-2 rat retina. *Am J Hypertens* **20**: 423–430.
- Chaturvedi, N, Porta, M, Klein, R, Orchard, T, Fuller, J, Parving, HH et al.; DIRECT Programme Study Group. (2008). Effect of candesartan on prevention (DIRECT-Prevent 1) and progression (DIRECT-Protect 1) of retinopathy in type 1 diabetes: randomised, placebo-controlled trials. *Lancet* **372**: 1394–1402.
- Sjölie, AK, Klein, R, Porta, M, Orchard, T, Fuller, J, Parving, HH et al.; DIRECT Programme Study Group. (2008). Effect of candesartan on progression and regression of retinopathy in type 2 diabetes (DIRECT-Protect 2): a randomised placebo-controlled trial. *Lancet* **372**: 1385–1393.
- Mauer, M, Zinman, B, Gardiner, R, Suissa, S, Sinaiko, A, Strand, T et al. (2009). Renal and retinal effects of enalapril and losartan in type 1 diabetes. *N Engl J Med* **361**: 40–51.
- Ghattas, A, Lip, PL and Lip, GY (2011). Renin-angiotensin blockade in diabetic retinopathy. *Int J Clin Pract* **65**: 113–116.
- Wright, AD and Dodson, PM (2010). Diabetic retinopathy and blockade of the renin-angiotensin system: new data from the DIRECT study programme. *Eye (Lond)* **24**: 1–6.
- Miyazaki, M and Takai, S (2006). Tissue angiotensin II generating system by angiotensin-converting enzyme and chymase. *J Pharmacol Sci* **100**: 391–397.
- Cristovam, PC, Arnoni, CP, de Andrade, MC, Casarini, DE, Pereira, LG, Schor, N et al. (2008). ACE-dependent and chymase-dependent angiotensin II generation in normal and glucose-stimulated human mesangial cells. *Exp Biol Med (Maywood)* **233**: 1035–1043.
- Kumar, R and Boim, MA (2009). Diversity of pathways for intracellular angiotensin II synthesis. *Curr Opin Nephrol Hypertens* **18**: 33–39.
- Shioya, N, Saegusa, Y, Nishimura, K and Miyazaki, M (1997). Angiotensin II-generating system in dog and monkey ocular tissues. *Clin Exp Pharmacol Physiol* **24**: 243–248.
- Nguyen, G, Delarue, F, Burcklé, C, Bouzir, L, Giller, T and Sraer, JD (2002). Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* **109**: 1417–1427.
- Ferreira, AJ, Santos, RA, Bradford, CN, Mecca, AP, Sumners, C, Katovich, MJ et al. (2010). Therapeutic implications of the vasoprotective axis of the renin-angiotensin system in cardiovascular diseases. *Hypertension* **55**: 207–213.
- Ferrario, CM, Trask, AJ and Jessup, JA (2005). Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol* **289**: H2281–H2290.
- Sica, DA (2002). The practical aspects of combination therapy with angiotensin receptor blockers and angiotensin-converting enzyme inhibitors. *J Renin Angiotensin Aldosterone Syst* **3**: 66–71.
- Perret-Guillaume, C, Joly, L, Jankowski, P and Benetos, A (2009). Benefits of the RAS blockade: clinical evidence before the ONTARGET study. *J Hypertens Suppl* **27**: S3–S7.
- Ribeiro-Oliveira, A Jr, Nogueira, AI, Pereira, RM, Boas, WW, Dos Santos, RA and Simões da Silva, AC (2008). The renin-angiotensin system and diabetes: an update. *Vasc Health Risk Manag* **4**: 787–803.
- Perkins, JM and Davis, SN (2008). The renin-angiotensin-aldosterone system: a pivotal role in insulin sensitivity and glycemic control. *Curr Opin Endocrinol Diabetes Obes* **15**: 147–152.
- Keidar, S, Kaplan, M and Gamliel-Lazarovich, A (2007). ACE2 of the heart: From angiotensin I to angiotensin (1-7). *Cardiovasc Res* **73**: 463–469.
- Iwai, M and Horieuchi, M (2009). Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis. *Hypertens Res* **32**: 533–536.
- Der Sarkissian, S, Huettelman, MJ, Stewart, J, Katovich, MJ and Raizada, MK (2006). ACE2: A novel therapeutic target for cardiovascular diseases. *Prog Biophys Mol Biol* **91**: 163–198.
- Huettelman, MJ, Grobe, JL, Vazquez, J, Stewart, JM, Mecca, AP, Katovich, MJ et al. (2005). Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. *Exp Physiol* **90**: 783–790.
- Hernández Prada, JA, Ferreira, AJ, Katovich, MJ, Shenoy, V, Qi, Y, Santos, RA et al. (2008). Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. *Hypertension* **51**: 1312–1317.
- Ferreira, AJ, Shenoy, V, Yamazato, Y, Sriramula, S, Francis, J, Yuan, L et al. (2009). Evidence for angiotensin-converting enzyme 2 as a therapeutic target for the prevention of pulmonary hypertension. *Am J Respir Crit Care Med* **179**: 1048–1054.
- Fraga-Silva, RA, Sorg, BS, Wankhede, M, Dedeugd, C, Jun, JY, Baker, MB et al. (2010). ACE2 activation promotes antithrombotic activity. *Mol Med* **16**: 210–215.

40. Der Sarkissian, S, Grobe, JL, Yuan, L, Narielwala, DR, Walter, GA, Katovich, MJ *et al.* (2008). Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology. *Hypertension* **51**: 712–718.
41. Li, Q, Verma, A, Han, PY, Nakagawa, T, Johnson, RJ, Grant, MB *et al.* (2010). Diabetic eNOS-knockout mice develop accelerated retinopathy. *Invest Ophthalmol Vis Sci* **51**: 5240–5246.
42. Huentelman, MJ, Zubcevic, J, Katovich, MJ and Raizada, MK (2004). Cloning and characterization of a secreted form of angiotensin-converting enzyme 2. *Regul Pept* **122**: 61–67.
43. Dawn-Linsley, M, Ekinci, FJ, Ortiz, D, Rogers, E and Shea, TB (2005). Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. *J Neurosci Methods* **141**: 219–222.
44. Nakagawa, T, Sato, W, Glushakova, O, Heinig, M, Clarke, T, Campbell-Thompson, M *et al.* (2007). Diabetic endothelial nitric oxide synthase knockout mice develop advanced diabetic nephropathy. *J Am Soc Nephrol* **18**: 539–550.
45. Ferrario, CM, Chappell, MC, Tallant, EA, Brosnihan, KB and Diz, DI (1997). Counterregulatory actions of angiotensin-(1-7). *Hypertension* **30**(3 Pt 2): 535–541.
46. Lorenz, JN (2010). Chymase: the other ACE? *Am J Physiol Renal Physiol* **298**: F35–F36.
47. Hauswirth WW, Li Q, Raisler B, Timmers AM, Berns KI, Flannery JG, LaVail MM and Lewin AS (2004). Range of retinal diseases potentially treatable by AAV-vectorized gene therapy. *Novartis Found Symp* **255**: 179–188; discussion 188–194.
48. Herzog, RW, Cao, O and Srivastava, A (2010). Two decades of clinical gene therapy—success is finally mounting. *Discov Med* **9**: 105–111.

ACE2 and Diabetic Complications

Rachael G. Dean and Louise M. Burrell*

Department of Medicine, University of Melbourne, Austin Health, Heidelberg 3081, Victoria, Australia

Abstract: Angiotensin converting enzyme (ACE) is a key enzyme in the renin angiotensin system (RAS) and converts angiotensin (Ang) I to the vasoconstrictor Ang II, which is thought to be responsible for most of the physiological and pathophysiological effects of the RAS. This classical view of the RAS was challenged with the discovery of the enzyme, ACE2 which both degrades Ang II and leads to formation of the vasodilatory and anti-proliferative peptide, Ang 1-7. Activation of the RAS is a major contributor to diabetic complications, and blockade of the vasoconstrictor and hypertrophic actions of Ang II, slows but does not prevent the progression of such complications. The identification of ACE2 in the heart and kidney adds further complexity to the RAS, provides the rationale to explore the role of this enzyme in pathophysiological states, including the microvascular and macrovascular complications of diabetes. It is believed that ACE2 acts in a counter-regulatory manner to ACE to modulate the balance between vasoconstrictors and vasodilators within the heart and kidney, and may thus play a significant role in the pathophysiology of cardiac and renal disease. Relatively little is known about ACE2 in diabetes, and this review will explore and discuss the data that is currently available. The discovery of ACE2 presents a novel opportunity to develop drugs that specifically influence ACE2 activity and/or expression, and it is possible that such compounds may have considerable clinical value in the prevention and treatment of the complications of diabetes.

Key Words: Diabetes mellitus, ACE2, renin angiotensin system, nephropathy, myocardial infarction, atherosclerosis, retinopathy.

INTRODUCTION

The pathophysiology of diabetic complications involves an interaction between haemodynamic and metabolic factors [1]. Relevant metabolic factors include glucose-dependent pathways such as advanced glycation, whereas haemodynamic factors include systemic hypertension, and vasoactive hormones, such as angiotensin II. Ang II is thought to be responsible for most of the physiological and pathophysiological effects of the RAS, and blockade of the RAS whether through inhibition of ACE which reduces formation of Ang II, or blockers of the Ang II type 1 (AT₁) receptor have been successful in the management of hypertension and heart failure, are standard therapy following myocardial infarction, and reduce the rate of progression of renal disease [2-5].

Until recently ACE was considered to be a key enzyme in the RAS. The classical view of the RAS has been challenged with the discovery of the enzyme, ACE2 [6,7] as well as the increasing awareness that many angiotensin peptides other than Ang II have biological activity and physiological importance [8]. The reported vasodilatory actions of Ang 1-7 [8], along with the potential involvement of ACE2 in both Ang II degradation and Ang 1-7 production adds another level of complexity to the RAS [9,10].

This review will examine how the "novel" aspects of the RAS, such as ACE2 and its vasodilatory product, Ang 1-7 may act as a counterbalance to the effects of ACE and Ang II. It will also detail how further investigation of these pathways will increase our understanding of the underlying pathogenic pathways involved in diabetic complications, and may lead to the development of new therapeutic approaches for the prevention and treatment of diabetes associated cardiac, vascular and renal disease.

THE CLASSICAL RENIN ANGIOTENSIN SYSTEM

Briefly, in the classic pathway of the RAS, renin is secreted from the juxtaglomerular apparatus of the kidney and acts on the circulating precursor angiotensinogen to generate Ang I (Fig. 1). Ang I is converted in the lungs by ACE, a dipeptidyl carboxypeptidase, to Ang II, a potent vasopressor, peptide that acts on the heart and the kidneys by binding to the G protein-coupled receptors AT₁

and AT₂. The AT₁ receptor mediates the deleterious effects of angiotensin II including vasoconstriction and trophic actions, whilst the AT₂ receptor regulates opposing effects.

NOVEL ASPECTS OF THE RAS

ACE2 is a newly described enzyme with a more restricted distribution than ACE, and expressed mainly in the heart and kidney. ACE2 was cloned from a human heart failure cDNA library [6] and a human lymphoma cDNA library [7] and is the first known human homologue of ACE. Analysis of the genomic sequence for ACE2 has identified that this gene contains 18 exons and maps to chromosomal location Xp22 [7]. The full length cDNA coding for ACE2 predicts a protein of 805 amino acids having 42% homology with the N-terminal catalytic domain of ACE, and a hydrophobic region near the C-terminus likely to serve as a membrane anchor. Like ACE, ACE2 is a type 1 membrane protein with the catalytic domain on the extracellular surface.

ACE2 has a much more restricted distribution than ACE. Gene expression of ACE2 was initially identified in cardiovascular tissues, kidney, and testis [6,7]. Cardiac ACE2 is predominantly localised to the endothelium and to a lesser extent the vascular smooth muscle cells of intra-cardiac vessels [6,11]. In the kidney, ACE2 is predominantly expressed in the proximal tubules [12], where it co-localises with Ang 1-7 [13]. Others have identified ACE2 in the retina [14], liver [15], gastrointestinal tract, brain, and lungs [16].

The precise physiological function of ACE2 is currently under intense investigation, and there is great interest in peptides which are either cleaved or generated by this enzyme. ACE2 removes a single C-terminal residue from Ang I to generate Ang 1-9, a peptide with no known function, and degrades Ang II to the biologically active peptide Ang 1-7 [6]. *In vitro* studies suggest that the catalytic efficiency of ACE2 for Ang II is 400-fold greater than for Ang I [17] indicating that the major role for ACE2 is the conversion of Ang II to Ang 1-7. Thus ACE2 may limit the vasoconstrictor action of Ang II through its inactivation, as well as counteracting the actions of Ang II through the formation of Ang 1-7.

In addition to effects on the angiotensin peptides, ACE2 cleaves the C-terminal residue of the peptides des-Arg⁹-bradykinin, neuropeptides 1-13 and kinetins [6], and hydrolyses apelin-13 and dynorphin A 1-13 with as high a catalytic efficiency as Ang II [17]. ACE2 has no effect on bradykinin in contrast to ACE emphasizing the specificity of ACE2 [6,17]. Many of the *in vitro* substrates of

*Address correspondence to this author at the Department of Medicine, University of Melbourne, Austin Health, Heidelberg 3081, Victoria, Australia; Tel: 61 3 9496 2159; Fax: 61 3 9497 4554; E-mail: l.burrell@unimelb.edu.au

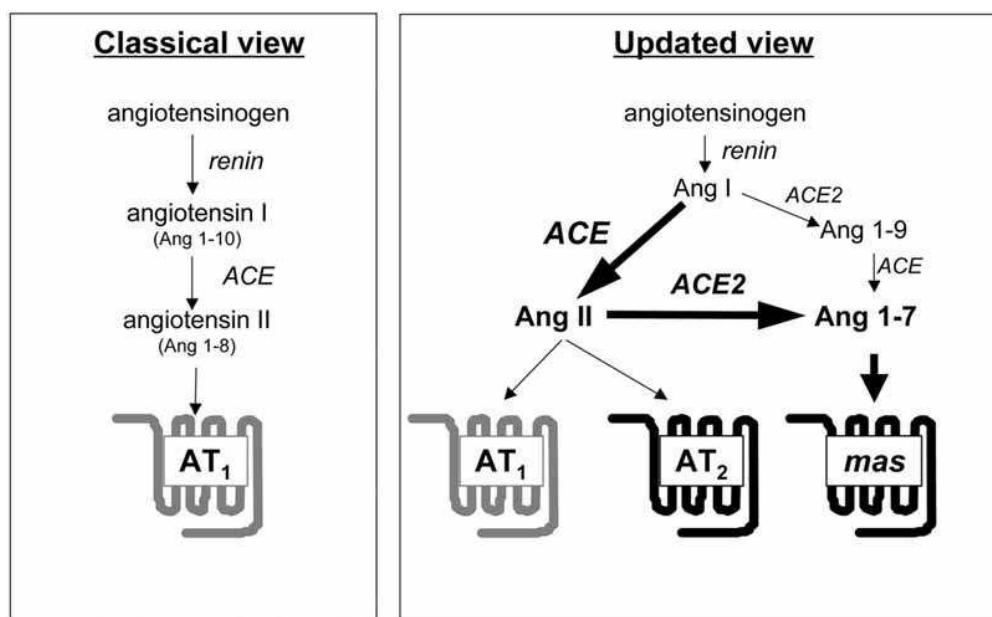


Fig. (1). The renin angiotensin system pathway.

The classical RAS shown on the right illustrates the main pathway for Ang II generation from Ang I via ACE, with effects being mediated via the G-protein coupled AT₁ receptor. The left hand panel shows the novel aspects of the RAS in which ACE2 plays a role to degrade Ang I to Ang 1-9, and Ang II to the vasodilator Ang 1-7. In this version, Ang II also mediates effects via the AT₂ receptor, whilst Ang 1-7 acts through the Mas receptor.

ACE2 have actions of relevance to cardiovascular regulation; apelin is a potent cardiac inotrope, dynorphin A is an endogenous opioid neuropeptide, and des-Arg9-bradykinin binds to the bradykinin B1 receptor, which is activated by inflammation and tissue injury [18]. It is not yet known if the *in vitro* substrates of ACE2 are also physiological *in vivo* substrates, and further studies are needed that address *in vivo* changes in the levels of putative substrates or products of ACE2 using ACE2 knockout [19], ACE2 transgenic animals [20], and ACE2 agonists as well as potent, selective ACE2 inhibitors [21,22]. The *in vitro* enzymatic activity of ACE2 is unaffected by ACE inhibitors [6,7] and although ACE2 inhibitors are available, there are no data as to the effect of these compounds *in vivo*.

As mentioned above, a major research focus concerns the actions of ACE2 on angiotensin peptides, and in particular Ang 1-7. The research work of Ferrario has been instrumental in expanding our knowledge of Ang 1-7, and he and colleagues have shown that Ang 1-7 has a potential role as a cardioprotective peptide with vasodilator, anti-growth and anti-proliferative actions [23,24]. Most recently the receptor for Ang 1-7 has been identified [25] and shown to be a G-protein-coupled receptor encoded by the Mas proto-oncogene, known as the Mas receptor. The functional significance of Mas is derived from studies where deletion of the Mas receptor abolished the binding of Ang 1-7 to kidney, and Mas deficient aortas were unable to relax in response to Ang 1-7 [25]. Others have reported that the Ang 1-7 dependent reduction in cardiomyocyte growth is mediated by the Mas receptor [26] and there is evidence of a functional interaction between the Mas receptor and the AT₁ and AT₂ receptors in the mouse heart [27].

DIABETES AND RENAL DISEASE

Diabetic nephropathy is the principal cause of end-stage renal failure in the Western world, and is a major cause of morbidity and mortality. Up to 20% of those with type 2 diabetes have diabetic kidney disease at the time of diagnosis, and a further 30-40% will develop diabetic nephropathy within 10 years of diagnosis. Alterations within the RAS are considered pivotal for the development of diabetic nephropathy [28] and blockade of the RAS provides significant renoprotection in both experimental models of diabetes and in man. ACE inhibitors were originally shown to retard the progres-

sion of type 1 diabetic nephropathy [29], and more recently angiotensin receptor blockade with losartan and irbesartan has been shown to retard the progression of type 2 diabetic nephropathy [2,3]. Hypertension is both a cause and a complication of renal disease, but the effects of RAS blockade to reduce proteinuria and retard progression appear to be independent of their blood pressure lowering effects [30].

To date there have been no human studies of the novel aspects of the RAS, and there is limited experimental data on ACE2. Both ACE and ACE2 protein are predominantly localised to the cortical tubules of the kidney [6,12], and a recent study has characterised ACE2 in the kidney of a rodent model of type 1 diabetes mellitus and compared and contrasted it to ACE [12]. Previous studies in the kidney using this model of diabetes demonstrated that ACE is down regulated in the renal tubules and upregulated in the glomerulus. As with ACE, ACE2 gene expression is also decreased in diabetic renal tubules by ~50% [12]. Deficiency in ACE2 would reduce Ang 1-7 formation, and allow Ang II accumulation, with consequent effects to cause tubulointerstitial fibrosis and renal failure. RAS blockade retards progression of renal damage, and interestingly, the reduction in ACE2 in the diabetic kidney was prevented by ACE inhibitor therapy, suggesting that ACE2 may indeed have a renoprotective role [12,31].

Support for this theory comes from studies in a model of type 2 diabetes, the spontaneously diabetic mouse (db/db) [32]. In this model, ACE2 expression was also examined in the kidney, but in contrast to the earlier study, ACE2 increased in the renal cortex. As these mice did not have overt nephropathy, the results raise the possibility that enhanced formation of Ang 1-7, subsequent to increased ACE2, balanced the deleterious actions of Ang II and prevented kidney damage. To test this hypothesis, studies that assess both ACE2 and Ang 1-7 levels during the time course of development of nephropathy are needed, as well as examination of the effects of long-term RAS blockade on these parameters.

DIABETES AND HYPERTENSION

Hypertension is common in patients with diabetes, the rate of progression of diabetic renal disease is closely correlated to blood pressure at baseline, and aggressive blood pressure lowering de-

creases the risk of macrovascular disease and death [33]. Most patients with diabetes need 3-4 agents to lower blood pressure to the targets recommended by international guidelines, and there appears to be particular advantage conferred by lowering blood pressure using RAS blockade. In the Losartan Intervention for Endpoint reduction in Hypertension (LIFE) trial, patients were randomised to either losartan or the beta-blocker atenolol; despite a similar fall in blood pressure with the 2 agents, only losartan reduced the combined endpoint of cardiovascular death, stroke or myocardial infarction [4]. Thus, although blood pressure reduction is a key component of the therapies, locally produced Ang II within the kidney or heart may have non-hemodynamic effects to cause cell hypertrophy, *via* induction of growth factors such as transforming growth factor beta or connective tissue growth factor [34,35], and thus blockade of the local, tissue RAS is also required to prevent cardiovascular morbidity and mortality.

It has been hypothesised that disruption of the ACE/ACE2 balance may result in abnormal blood pressure, with increased ACE2 expression protecting against hypertension, and ACE2 deficiency causing hypertension [36]. Several lines of evidence do support a role for ACE2 in hypertension. For example, ACE2 gene expression is inversely related to blood pressure in experimental models of hypertension [19], transgenic mice that overexpress ACE2 have lower blood pressure, and in several rat models of experimental hypertension, the gene for ACE2 maps to a defined quantitative trait locus on the X chromosome previously identified as a quantitative locus for blood pressure [19]. In addition, an association between ACE2 and blood pressure has been reported; in the spontaneously hypertensive rat (SHR) and SHR rat-stroke prone rats, renal ACE2 mRNA levels are reduced compared to normotensive Wistar-Kyoto rats [19]. It however remains to be clarified whether ACE2 deficiency has a pathophysiological role in the onset of hypertension or is simply a consequence of elevated blood pressure.

DIABETES AND HEART DISEASE

Cardiovascular disease is the major cause of morbidity and mortality in patients with diabetes [37]. The pathophysiology for this increased risk involves traditional and novel cardiac risk factors including hypertension, dyslipidaemia, smoking, hyperglycaemia, metabolic abnormalities, endothelial dysfunction, autonomic dys-

function, obesity, renal failure and anaemia [38,39]. Diabetic patients with cardiovascular disease may present with coronary artery disease, heart failure and/or sudden cardiac death.

Improved understanding of the underlying morphological changes that occur in the diabetic heart disease, or diabetic cardiomyopathy heart may lead to improved therapies. Some of the early changes in both human and experimental models include myocyte hypertrophy, perivascular fibrosis and accumulation of extracellular matrix protein and advanced glycation end-products [34]. In addition, the central role of the RAS in the cardiac complication of diabetes is evident from the results of RAS blockade trials which reduce cardiovascular events in those with diabetes. As mentioned, in patients with hypertension, diabetes, and left ventricular hypertrophy, Ang II receptor blockade with losartan was more effective than atenolol in reducing cardiovascular morbidity and mortality [4]. These data suggests that RAS blockade in diabetes may have benefits beyond blood pressure reduction alone.

The role of ACE2 in diabetic cardiac complications has not been examined in any detail, but its localisation in the heart suggests an important role for cardiovascular function. Certainly, ACE2 knockout mice, which lack ACE2 protein [19] develop abnormal heart function with severely impaired cardiac contractility. The importance of ACE2 in cleaving and/or inactivating Ang II is shown by the increase in plasma, cardiac and kidney Ang II levels in the knockout mice, and confirmed by studies in which the genetic ablation of ACE on an ACE2 mutant background completely rescued the cardiac phenotype [19]. The hearts of the ACE2 knockout mice also had upregulation of hypoxia markers, suggesting reduced oxygen delivery to the myocytes [19]. As ACE2 is localised to endothelial cells of intramyocardial vessels and smooth muscle cells in both rat and man [11], it is likely that ACE2 plays a role in the control of local vasodilation in the heart.

There is limited data available on ACE2 in the diabetic heart. We have previously shown in the streptozotocin diabetic heart, that there is increased left ventricular mass and decreased left ventricular collagen solubility, and increased collagen III gene and protein expression [34]. Our recent studies have assessed the expression of ACE2 in the hearts of diabetic rats, and preliminary findings indicate down regulation of cardiac ACE2 gene expression [40], a finding confirmed at the protein level (Fig. 2). Such changes parallel

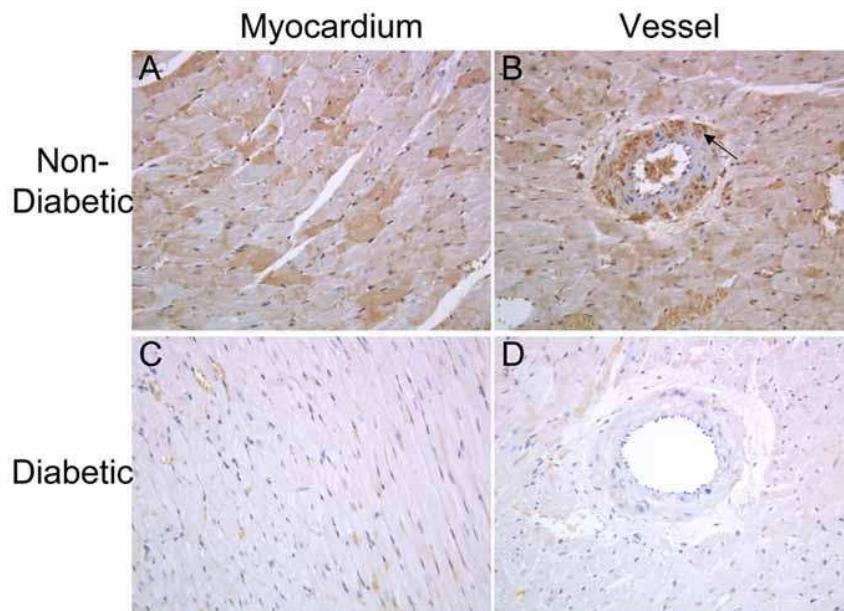


Fig. (2). Photomicrographs showing immunohistochemical localisation of ACE2 protein in the myocardium (A and C) and arteries (B and D) from non-diabetic control (A and B) and diabetic (C and D) rat left ventricle. ACE2 protein is localised to cardiac myocytes (A) and artery smooth muscle cells (arrow) (B). There is less immunostaining in the left ventricles from diabetic animals. Magnification X500.

those observed in the diabetic kidney, and suggest that dysregulation of ACE2 occurs in both the heart and the kidney in experimental diabetes.

Diabetes is often associated with left ventricular hypertrophy, which is a risk factor for cardiovascular morbidity and mortality, and is often associated with diastolic dysfunction. Recent studies have shown that lentiviral delivery of ACE2 protects against cardiac hypertrophy and myocardial fibrosis induced by Ang II infusion in the rat [41]. In deoxycorticosterone acetate-salt hypertension, chronic Ang 1-7 infusion also prevented cardiac fibrosis independently of changes in blood pressure or cardiac hypertrophy [42].

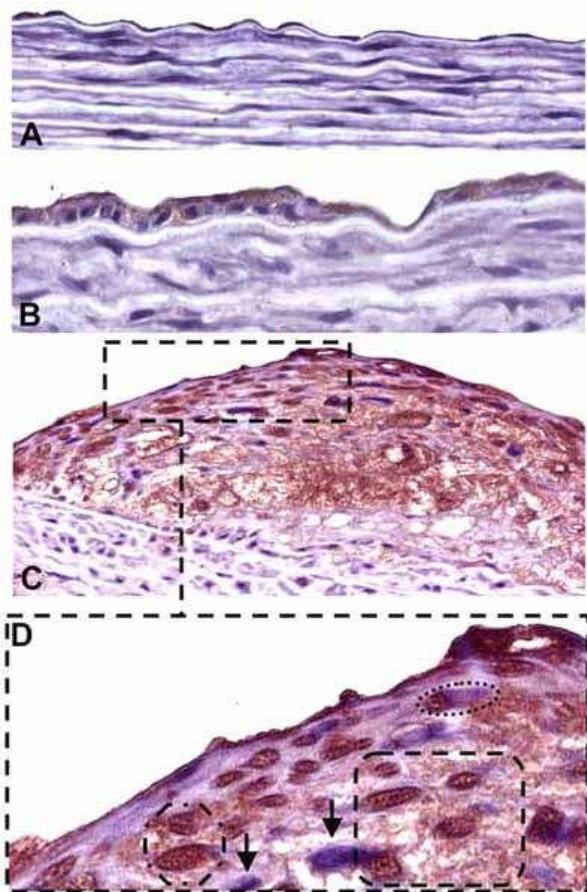


Fig. (3). Photomicrographs of sections of rabbit thoracic aorta showing immunolocalisation of ACE2 protein. ACE2 immunostaining was not present in the normal thoracic aorta endothelium (A), but clearly visible in the neointima (B) and plaques (C and D). In plaques, ACE2 positive cells were abundant (C) and in the magnified area (D) myofibroblasts show ACE2 immunoreactivity. Magnification: A and B; X300, C; X200 and D; X400. (Reproduced, with permission, from Zulli A. et al., Journal of Histochemistry and Cytochemistry, 54, 2:147-150, 2006).

ATHEROSCLEROSIS AND CORONARY ARTERY DISEASE

Coronary artery disease is a major contributor to early death in many subjects with type 2 diabetes, and patients with diabetes have accelerated, aggressive and diffuse coronary artery disease. Diabetes itself is considered a coronary heart disease equivalent as it confers the same increase in cardiovascular risk as a previous myocardial infarction in a non-diabetic subject [43] and is associated with a 3-5-fold increase in mortality.

The vascular RAS plays a pivotal role in the development and acceleration of atherosclerosis in diabetes. In an elegant study in a model of diabetes-induced plaque formation in the apolipoprotein E-null mouse [44], which have significant increases in aortic AT₁ receptor expression, atherosclerosis was ameliorated by AT₁ receptor blockade but not by calcium channel antagonism. Recently, we have shown very high expression of ACE2 in macrophages and α -smooth muscle cells within atherosclerotic plaques in a rabbit model of atherosclerosis [45] (Fig. 3). It is not clear whether the increase is a response to injury, to increase levels of Ang 1-7, and convert Ang II to Ang 1-7 via ACE2. As yet, there are no reported intervention studies in atherosclerosis that have addressed ACE2, and it is unknown if deficiency of ACE2 may contribute to the accelerated atherosclerotic disease process seen in diabetes.

The consequence of atherosclerosis is myocardial infarction. There is significant activation of the cardiac RAS after coronary artery occlusion [46], and it is well known that RAS blockade reduces remodelling and improves survival in man after a myocardial infarction. In recent studies [11], cardiac ACE2 expression and activity are also increased with experimental myocardial infarction, and ACE2 is highly expressed in infiltrating mononuclear cells, suggesting that it participates in the initial inflammatory response to injury. As ACE2 immunoreactivity was also observed in endothelial cells and myocytes some time after injury, ACE2 may be involved in the late phase of post myocardial infarction changes when injury and inflammation have abated but mechanical load remains high and the non-infarcted myocardium is undergoing a complex series of molecular and cellular events that lead to changes in the shape and function of the myocardium [47]. ACE2 protein expression is also increased in ischaemic cardiac explants of heart transplant recipients [11], and upregulated in human idiopathic cardiomyopathy [48].

These data suggest that increased ACE2 may limit the adverse effects of elevated ACE and Ang II in the heart by increasing levels of Ang 1-7. This is partly confirmed by an elegant study of 14 subjects with idiopathic cardiomyopathy, where an increase in functional cardiac ACE2 activity assessed by the ex-vivo formation of Ang 1-7 was reported [49]. In the rat infarct model, the development of heart failure was associated with increased expression of Ang 1-7 immunoreactivity, particularly in the myocytes surrounding the infarct region [50]. The functional significance of changes in Ang 1-7 is shown by studies in which infusion of Ang 1-7 attenuated the development of heart failure after MI [51] and reversed arrhythmia reperfusion injury [52].

RETINOPATHY

Retinopathy is the most common complication of diabetes, and one of the leading causes of blindness in people of working age [53]. RAS activation is strongly implicated in the development of diabetic retinopathy and RAS blockade partially attenuates pathologic changes in the diabetic retina [54]. ACE2 is also expressed in the retina, in the macroglial Müller cells which are an important site for the development of retinopathy [14]. Interestingly, in diabetic rats, ACE2 expression was increased in the retina compared to non-diabetic controls [14]. This result may reflect that there is only moderate retinopathy in this model, and by analogy to the changes observed in the kidney of type 2 diabetic mice, it is possible that the increase in ACE2 is playing a role to prevent significant injury. Again, studies are needed that trace the time course of changes in both ACE and ACE2, and their products, Ang II and Ang 1-7 and to correlate these changes with the degree of injury.

CONCLUSION

The renin angiotensin system has proved to be an important regulator of cardiovascular and renal structure and function. Activation of the RAS is a major contributor to diabetic complications, and blockade of the vasoconstrictor and hypertrophic actions of Ang II slows but does not prevent the progression of such compli-

cations. The identification of ACE2 in the heart, blood vessels and the kidney, its modulation in heart failure, atherosclerosis, retinopathy, hypertension and renal disease and evidence that this enzyme plays a biological role in the generation and degradation of vasodilatory and anti-trophic angiotensin peptides provides a rationale to further explore the role of this enzyme in pathophysiological states, including the microvascular and macrovascular complications of diabetes. The discovery of ACE2 also provides a novel opportunity to develop drugs that specifically influence its activity and/or expression, as it is likely that such compounds may have considerable clinical value in the prevention and treatment of the complications of diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia, and the National Heart Foundation of Australia.

ABBREVIATIONS

ACE	= Angiotensin converting enzyme
RAS	= Renin angiotensin system
Ang	= Angiotensin
AT ₁	= Ang II type 1
SHR	= Spontaneously hypertensive rat

REFERENCES

- References 55-57 are related articles recently published in Current Pharmaceutical Design.
- [1] Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet* 1998; 352: 213-9.
 - [2] Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001; 345: 861-9.
 - [3] Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, et al. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 2001; 345: 851-60.
 - [4] Lindholm LH, Ibsen H, Dahlof B, Devereux RB, Beevers G, de Faire U, et al. Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* 2002; 359: 1004-10.
 - [5] Johnston CI. Tissue angiotensin converting enzyme in cardiac and vascular hypertrophy, repair, and remodeling. *Hypertension* 1994; 23: 258-68.
 - [6] Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 2000; 87: E1-E9.
 - [7] Tippins SR, Hooper NM, Hyde R, Karra E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme - cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 2000; 275: 33238-43.
 - [8] Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counterregulatory actions of angiotensin-(1-7). *Hypertension* 1997; 30: 535-41.
 - [9] Turner AJ. Exploring the structure and function of zinc metallopeptidases: old enzymes and new discoveries. *Biochem Soc Trans* 2003; 31: 723-7.
 - [10] Oudit GY, Crackower MA, Backx PH, Penninger JM. The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc Med* 2003; 13: 93-101.
 - [11] Burrell LM, Risvanis J, Kubota E, Dean RG, MacDonald PS, Lu S, et al. Myocardial infarction increases ACE2 expression in rat and humans. *Eur Heart J* 2005; 26: 369-75; discussion 322-4.
 - [12] Tikellis C, Johnston CI, Forbes JM, Burns WC, Burrell LM, Risvanis J, et al. Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* 2003; 41: 392-7.
 - [13] Brosnihan KB, Neves LA, Joyner J, Averill DB, Chappell MC, Sarao R, et al. Enhanced renal immunocytochemical expression of ANG-(1-7) and ACE2 during pregnancy. *Hypertension* 2003; 42: 749-53.
 - [14] Tikellis C, Johnston CI, Forbes JM, Burns WC, Thomas MC, Lew RA, et al. Identification of angiotensin converting enzyme 2 in the rodent retina. *Curr Eye Res* 2004; 29: 419-27.
 - [15] Paizis G, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, et al. Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* 2005; 54: 1790-6.
 - [16] Harmer D, Gilbert M, Borman R, Clark K. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett* 2002; 532: 107-10.
 - [17] Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2002; 277: 14838-43.
 - [18] Duka I, Kintsurashvili E, Gavras I, Johns C, Bresnahan M, Gavras H. Vasoactive potential of the B-1 bradykinin receptor in normotension and hypertension. *Circulation Research* 2001; 88: 275-81.
 - [19] Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, et al. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 2002; 417: 822-8.
 - [20] Donoghue M, Wakimoto H, Maguire CT, Acton S, Hales P, Stagliano N, et al. Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins. *J Mol Cell Cardiol* 2003; 35: 1043-53.
 - [21] Huang L, Sexton DJ, Skogerson K, Devlin M, Smith R, Sanyal I, et al. Novel peptide inhibitors of angiotensin-converting enzyme 2. *J Biol Chem* 2003; 278: 15532-40.
 - [22] Dales NA, Gould AE, Brown JA, Calderwood EF, Guan B, Minor CA, et al. Substrate-based design of the first class of angiotensin-converting enzyme-related carboxypeptidase (ACE2) inhibitors. *J Am Chem Soc* 2002; 124: 11852-3.
 - [23] Ferrario CM. Angiotensin-converting enzyme 2 and angiotensin-(1-7): an evolving story in cardiovascular regulation. *Hypertension* 2006; 47: 515-21.
 - [24] Tallant EA, Clark MA. Molecular mechanisms of inhibition of vascular growth by angiotensin-(1-7). *Hypertension* 2003; 42: 574-9.
 - [25] Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 2003; 100: 8258-63.
 - [26] Tallant EA, Ferrario CM, Gallagher PE. Angiotensin-(1-7) inhibits growth of cardiac myocytes through activation of the mas receptor. *Am J Physiol Heart Circ Physiol* 2005; 289: H1560-6.
 - [27] Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, Almeida AP. Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension* 2005; 46: 937-42.
 - [28] Cooper ME, Johnston CI. Optimizing treatment of hypertension in patients with diabetes. *JAMA* 2000; 283: 3177-9.
 - [29] Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med* 1993; 329: 1456-62.
 - [30] Brenner BM. Retarding the progression of renal disease. *Kidney Int* 2003; 64: 370-8.
 - [31] Ferrario CM. Commentary on Tikellis et al: There is more to discover about angiotensin-converting enzyme. *Hypertension* 2003; 41: 390-1.
 - [32] Ye M, Wysocki J, Naaz P, Salabat MR, LaPointe MS, Battie D. Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? *Hypertension* 2004; 43: 1120-5.
 - [33] Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. UK Prospective Diabetes Study Group. *BMJ* 1998; 317: 703-13.
 - [34] Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC, et al. A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res* 2003; 92: 785-92.

- [35] Dean RG, Balding LC, Candido R, Burns WC, Cao Z, Twigg SM, *et al.* Connective tissue growth factor and cardiac fibrosis after myocardial infarction. *J Histochem Cytochem* 2005; 53: 1245-56.
- [36] Yagil Y, Yagil C. Hypothesis: ACE2 modulates blood pressure in the mammalian organism. *Hypertension* 2003; 41: 871-3.
- [37] Candido R, Srivastava P, Cooper ME, Burrell LM. Diabetes mellitus: a cardiovascular disease. *Curr Opin Investig Drugs* 2003; 4: 1088-94.
- [38] Grundy SM, Howard B, Smith S, Jr., Eckel R, Redberg R, Bonow RO. Prevention Conference VI: Diabetes and Cardiovascular Disease: executive summary: conference proceeding for healthcare professionals from a special writing group of the American Heart Association. *Circulation* 2002; 105: 2231-9.
- [39] Srivastava PM, Thomas MC, Calafiore P, Maclsaac RJ, Jerums G, Burrell LM. Diastolic dysfunction is associated with anaemia in patients with Type II diabetes. *Clin Sci (Lond)* 2006; 110: 109-16.
- [40] Dean RG, Nordkamp M, Burchill LJ, Griggs K, Tikellis C, Lu S, *et al.* ACE2, a target for diabetic heart disease? Heart Foundation Conference and Scientific Meeting. Sydney, NSW, 2006; 23.
- [41] Huentelman MJ, Grobe JL, Vazquez J, Stewart JM, Mecca AP, Katovich MJ, *et al.* Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. *Exp Physiol* 2005; 90: 783-90.
- [42] Grobe JL, Mecca AP, Mao H, Katovich MJ. Chronic angiotensin-(1-7) prevents cardiac fibrosis in the DOCA-salt model of hypertension. *Am J Physiol Heart Circ Physiol* 2006; 290(6): H2417-23.
- [43] Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998; 339: 229-34.
- [44] Candido R, Allen TJ, Lassila M, Cao Z, Thallas V, Cooper ME, *et al.* Irbesartan but not amlodipine suppresses diabetes-associated atherosclerosis. *Circulation* 2004; 109: 1536-42.
- [45] Zulli A, Burrell LM, Widdop RE, Black MJ, Buxton BF, Hare DL. Immunolocalization of ACE2 and AT2 receptors in rabbit atherosclerotic plaques. *J Histochem Cytochem* 2006; 54: 147-50.
- [46] Duncan AM, Burrell LM, Campbell DC. Effects of angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptides in rats with myocardial infarction. *J Cardiovasc Pharmacol* 1996; 28: 746-54.
- [47] Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. *J Am Coll Cardiol* 2000; 35: 569-82.
- [48] Goulter AB, Goddard MJ, Allen JC, Clark KL. ACE2 gene expression is up-regulated in the human failing heart. *BMC Med* 2004; 2: 19.
- [49] Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR, *et al.* Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. *Circulation* 2003; 108: 1707-12.
- [50] Averill DB, Ishiyama Y, Chappell MC, Ferrario CM. Cardiac angiotensin-(1-7) in ischemic cardiomyopathy. *Circulation* 2003; 108: 2141-6.
- [51] Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, *et al.* Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation* 2002; 105: 1548-50.
- [52] Santos RA, Ferreira AJ, Nadu AP, Braga AN, de Almeida AP, Campagnole-Santos MJ, *et al.* Expression of an angiotensin-(1-7)-producing fusion protein produces cardioprotective effects in rats. *Physiol Genomics* 2004; 17: 292-9.
- [53] Sjolie AK, Chaturvedi N. The retinal renin-angiotensin system: implications for therapy in diabetic retinopathy. *J Hum Hypertens* 2002; 16(Suppl 3): S42-6.
- [54] Chaturvedi N, Sjolie AK, Stephenson JM, Abrahamian H, Keipes M, Castellarin A, *et al.* Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. The EUCLID Study Group. *EURODIAB Controlled Trial of lisinopril in insulin-dependent diabetes mellitus*. *Lancet* 1998; 351: 28-31.
- [55] Luno J, Praga M, de Vinuesa SG. The reno-protective effect of the dual blockade of the renin angiotensin system (RAS). *Curr Pharm Des* 2005; 11(10): 1291-300.
- [56] Onder G, Vedova CD, Pahor M. Effects of ACE inhibitors on skeletal muscle. *Curr Pharm Des* 2006; 12(16): 2057-64.
- [57] Shimada Y. Does Angiotensin converting enzyme inhibitor protect the heart in cardiac surgery? From laboratory to operating room: clinical application of experimental study. *Curr Pharm Des* 2006; 12(4): 517-26.

Prediction of Off-Target Effects on Angiotensin-Converting Enzyme 2

LIDIA V. KULEMINA¹ and DAVID A. OSTROV²

The authors describe a structure-based strategy to identify therapeutically beneficial off-target effects by screening a chemical library of Food and Drug Administration (FDA)-approved small-molecule drugs matching pharmacophores defined for specific target proteins. They applied this strategy to angiotensin-converting enzyme 2 (ACE2), an enzyme that generates vasodilatory peptides and promotes protection from hypertension-associated cardiovascular disease. The conformation-based structural selection method by molecular docking using DOCK allowed them to identify a series of FDA-approved drugs that enhance catalytic efficiency of ACE2 *in vitro*. These data demonstrate that libraries of approved drugs can be rapidly screened to identify potential side effects due to interactions with specific proteins other than the intended targets. (*Journal of Biomolecular Screening* 2011;16:878-885)

Key words: computational chemistry, compound repositories, chemoinformatics, general pharmaceutical process, cardiac diseases

INTRODUCTION

HERE IS A WELL-ESTABLISHED PARADIGM FOR TRANSLATION of small molecules into therapeutic agents.¹⁻³ This process consists of identification of lead compounds that are elaborated until a sufficient level of efficacy and safety is established for human clinical trials.⁴⁻⁶ Studies show that clinical trials for treatment of chronic conditions such as hypertension typically require 6 to 7 years to obtain statistically significant data regarding the safety of new drugs.⁷⁻¹⁰

We previously used *in silico* docking to identify the compounds capable of enhancing catalytic activity of Angiotensin-Converting enzyme 2 (ACE2) to lower blood pressure and prevent cardiovascular disease.^{11,12} We used DOCK5.1 (University of California, San Francisco [UCSF]) to explore a structural feature in the hinge region of ACE2 (site 1, Fig. 1) that is implicated in conformational shuffling between the two isoforms of the enzyme.¹³ A small molecule library of 139,735 compounds (molecular weight less than 500 Da) from National

Cancer Institute (NCI) Developmental Therapeutics program plated set collection was docked into the selected site 1 and scored using grid-based scoring system. Compounds were ranked according to their combined energy scores for hydrogen bonding and van der Waals contact interactions. The highest scoring compounds were obtained and assayed *in vitro* for their abilities to enhance ACE2 catalytic activity. One of the compounds, [8-(2-dimethylaminoethylamino)-5-(hydroxymethyl)-9-oxoanthen-2-yl]4-methyl-benzenesulfonate (from here on referred to as XNT) was shown to enhance the rate of catalysis by increasing the velocity of the enzyme approximately twofold (13). In vivo administration of XNT in the spontaneous hypertensive rat model induced a significant (71 ± 9 mm Hg) decrease in high blood pressure and resulted in improvements in cardiac function, including reversal of myocardial, perivascular, and renal fibrosis.¹⁴

Because the molecular docking selection strategy was useful in identifying compounds that enhance ACE2 catalytic activity and lower blood pressure *in vivo*, in this study we applied this selection strategy to a chemical library of 1217 Food and Drug Administration (FDA)-approved compounds with extensive information on bioavailability, toxicity, and safety (Distributed Structure-Searchable Toxicity Database Network, www.epa.gov/ncct/dsstox). Here we report identification of specific FDA-approved compounds that enhance the catalysis of ACE2. This rapid and economical molecular docking approach can be applied to other clinically relevant proteins to explore their structural features and identify off-target effects of the small molecule compounds.

¹Department of Chemistry, University of Florida, Gainesville.

²Department of Pathology, University of Florida, Gainesville.

Received Nov 22, 2010, and in revised form Feb 17, 2011. Accepted for publication Apr 15, 2011.

Supplementary material for this article is available on the *Journal of Biomolecular Screening* Web site at <http://jbx.sagepub.com/supplemental>.

Journal of Biomolecular Screening 16(8); 2011

DOI: 10.1177/1087057111413919

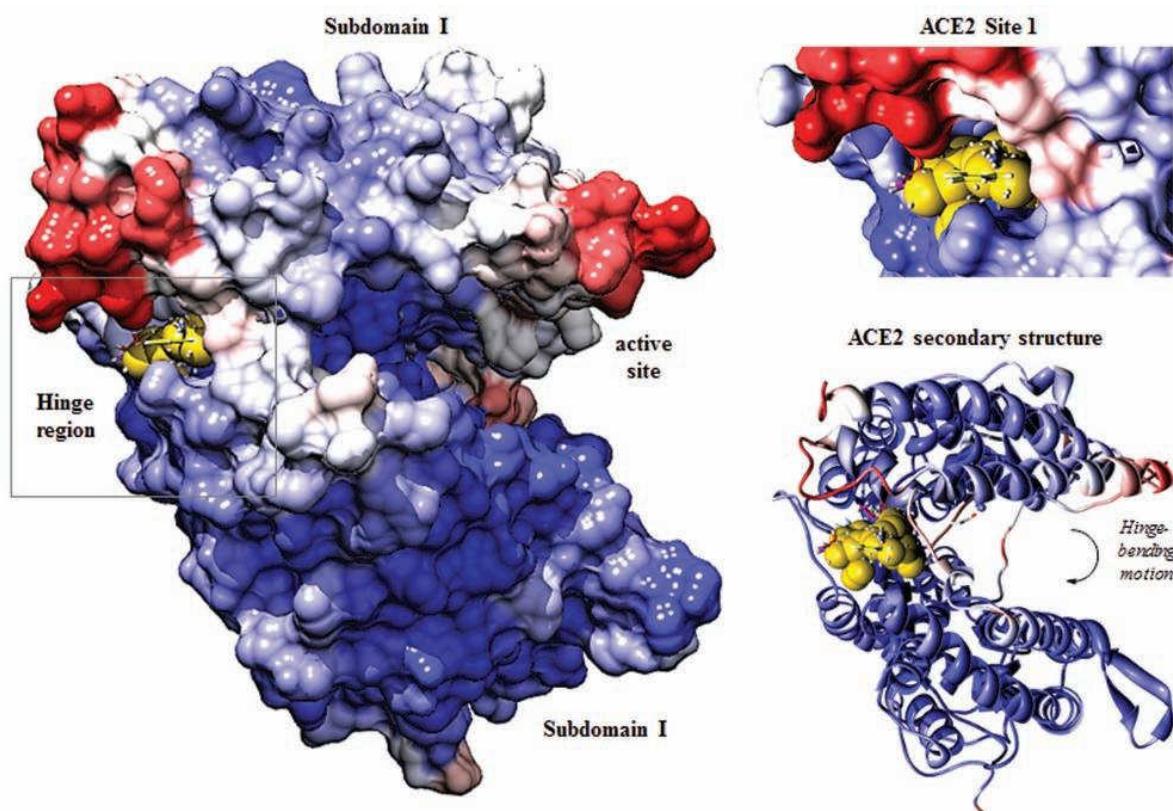


FIG. 1. Angiotensin-converting enzyme 2 (ACE2) site 1 used as a structural basis for molecular docking of Food and Drug Administration-approved compounds. Site 1, located in the hinge region separating the two subdomains of the enzyme, is characterized by high solvent accessibility and flexibility. Molecular surface and secondary structure of ACE2 in the open conformation (PDB ID: 1R42) are colored by accessible surface area (ASA): red for the highest and blue the lowest ASA. Yellow spheres represent the site selected for *in silico* screening; 10 highest scoring small molecules are shown in their predicted binding positions and represented as sticks. Inset shows zoomed-in view of the site selected for molecular docking.

MATERIALS AND METHODS

Molecular docking

We used the crystal structure of the apo form of human ACE2 in the open conformation (PDB ID: 1R42) to provide the basis for molecular docking.¹⁵ To prepare the site for docking, all water molecules have been removed. Protonation of ACE2 residues was done with SYBYL.¹⁶ Intermolecular assisted model building and energy refinement (AMBER) energy scoring (van der Waals + coulombic), contact scoring, and bump filtering were done in DOCK5.1.0.^{17,18} Atomic coordinates for 1,217 FDA-approved compounds were obtained from the ZINC structural database (<http://zinc.docking.org>). Each of the molecules was positioned into the selected site (Site 1) in 1000 different orientations and scored with rank based on the

predicted polar (H-bonding) and nonpolar (van der Waals) interactions. Compounds were selected to include protonation variants at medium pH (5.75–8.25). The scoring grid was set at 5 Å around the spheres selected for molecular docking. Molecular surface of the structure was explored using sets of spheres to describe potential binding pockets. The sites selected for molecular docking were defined using the SPHGEN program and filtered through the CLUSTER program.¹⁸ The SPHGEN program generates sets of overlapping spheres to describe the shape of the selected site. The CLUSTER program groups the selected spheres to define the points that are used by DOCK to match (superimpose) potential ligand atoms with spheres. Intermolecular AMBER energy scoring (van der Waals + coulombic), contact scoring, and bump filtering were implemented in DOCK v5.1.0. UCSF Chimera software package²⁰ was used to generate molecular graphic

images. The UF High-Performance Computing Center linux cluster was used to run docking jobs by parallel processing. The 38 highest scoring compounds (based on the combined contact and electrostatic score) were obtained from NCI Developmental Therapeutics program for testing in ACE2 catalytic assays.

ACE2 enzyme kinetic assays

Effects of 40 top-scoring compounds selected in virtual screening were tested in fluorescence-based kinetic assays using recombinant human enzyme and fluorogenic peptide substrate. Kinetic parameters for ACE2 in the presence of selected compounds were determined under steady-state conditions in the presence of saturating amounts of the substrate. Enzyme concentration was adjusted to ensure that <15% of the substrate was consumed at the lowest substrate concentration and product formation was linear with time. ACE2 assays for catalytic activity were carried out in a total volume of 100 μL , containing 75 mM Tris-HCl (pH 7.4), 0.1M NaCl, 0.5 μM ZnCl₂, 10 nM ACE2, and 0.01% Triton-X. Small-molecule stocks were prepared by dissolving the compounds in DMSO to a concentration of 50 to 100 mM; a final concentration of 50 μM was used in all screening experiments. Compounds were preincubated in black 96-well plates with 10 nM human recombinant ACE2 (Enzo Life Sciences, Plymouth Meeting, PA) for 15 min at 37 °C. Reactions were initiated by addition of 25 to 250 μM fluorogenic peptide substrate Mca-APK(Dnp)-OH (AnaSpec, Fremont, CA) and monitored continuously for 30 min with Spectra Max Gemini M5 Fluorescence Reader from Molecular Devices (Sunnyvale, CA; $\lambda_{\text{excitation}} = 325 \text{ nm}$, $\lambda_{\text{emission}} = 395 \text{ nm}$). Initial velocities of ACE2 in the absence and in presence of 50- μM compounds were determined by measuring an increase in fluorescence upon hydrolysis of the substrate. Nonlinear regression analysis and fitting of the data into Michaelis–

$V_o = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]}$ Menten were done with SigmaPlot software (Systat Software, San Jose, CA). Turnover numbers (k_{cat}) were calculated from the equation $k_{\text{cat}} = \frac{V_{\text{max}}}{[E]}$. EC₅₀ values for diminazene (DMZ) were determined by fitting initial velocity data for ACE2 in the presence of varying concentrations of DMZ and 50 μM Mca-APK(Dnp)-OH into a nonlinear regression model for the four-parameter logistic Hill equation

$E_0 + \frac{E_{\text{max}} \cdot C^\alpha}{EC_{50}^\alpha + C^\alpha}$, where E corresponds to effect (initial velocity) at a given substrate concentration, E_{max} is the maximal velocity measured, C is the drug concentration, and α is the Hill coefficient of sigmoidicity.

Inner filter effect

Inherent small-molecule fluorescence may alter apparent initial velocities, k_{cat} and K_m , as a result of inner filter effect (IFE). IFE is a reduction in fluorescence signal due to absorption of the exciting light and/or emitted radiation in the presence of a second fluorophore. As a result, only a fraction of the photons reach the fluorophores and get picked up by the detection system. For this reason, we corrected observed fluorescence

(F_{obs}) for IFE using the following equation: $F_{\text{observed}} = \frac{F_{\text{corrected}}}{IFE}$, where $FE = f_{\text{ex}} \cdot f_{\text{em}} = 10^{(A_{\text{ex}} + A_{\text{em}})/4}$, as described in Palmier and Van Doren.²¹

High-performance liquid chromatography analysis of Ang II cleavage

ACE2 activity assays were carried out in a total volume of 100 μL , containing 100 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.5 μM ZnCl₂; 10 nM ACE2 was preincubated for 5 min with 50 μM DMZ, and the reaction was initiated by addition of 50 μM substrate. Reactions were monitored for the first 15 min at $\lambda_{\text{ex}} = 328 \text{ nm}$, $\lambda_{\text{em}} = 392$ and stopped after 2 h at 37 °C by adding 10 μL of 0.5 M EDTA, followed by heating to 100 °C for 2 min. Samples were centrifuged at 11,600 x g for 10 min, supernatants were collected and concentrated under vacuum. The resulting residue was resuspended in 50 μL of the mobile phase B and applied to a C18 reverse-phase high-performance liquid chromatography (HPLC) column (column: Bondclone 10 μm , 150 × 2.1 mm [Phenomenex, Torrance, CA]; HPLC system: Agilent 1100 series [Agilent Technologies, Santa Clara, CA]) with a UV detector set at 214 nm. A linear solvent gradient of 11% to 100% B over 15 min with 10 min at final conditions and 10 min reequilibration was used. Mobile phase A consisted of 0.08% (v/v) phosphoric acid, and mobile phase B consisted of 40% (v/v) acetonitrile in 0.08% (v/v) phosphoric acid. The peaks corresponding to full-length peptides and peptide hydrolysis products were compared with the standards, and peak areas were integrated to calculate the extent of hydrolysis. Peak identities were determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectrometry using Applied Biosystems Voyager System 6031 (Applied Biosystems, Foster City, CA). Ang (1–7) resolved with a characteristic peak at m/z = 899.65, Ang II at m/z = 1046.52.²²

Statistical significance

Data are expressed as mean ± SD. Unpaired Student's *t* test and one-way analysis of variance (ANOVA) were performed for statistical analysis. Differences were considered significant at $p < 0.01$ or $p < 0.001$, as indicated. All statistical analysis and curve fitting were performed with SigmaPlot 11.0 from Systat Software.

Prediction of Off-Target Effects on ACE2

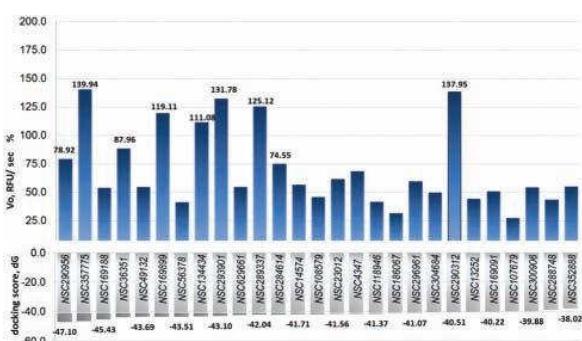


FIG. 2. Docking scores and measured initial velocities for the highest scoring 27 compounds. Angiotensin-converting enzyme 2 (ACE2) experimental initial velocities were determined in the presence of 38 top-scoring Food and Drug Administration-approved compounds (50 μM) and compared to those in the absence of the drugs. Compounds are shown ranked by combined energy score (gray bars). Dark blue bars demonstrate change in initial velocity for the first 27 compounds, where $\%V_0 = \frac{V_0 \text{ drug}}{V_0 \text{ no drug}} \cdot 100\%$ and 100% represents no change. Nine compounds with the highest initial velocities identified in this screening were selected for further testing.

RESULTS

Molecular docking of FDA approved drugs

In the previous study, we used molecular docking to target three structural features on the solvent-accessible surface of ACE2 and used *in vitro* assays to evaluate the effect of selected small molecules on enhancing catalytic activity.¹³ Small molecules targeted at the ACE2 hinge region, (Site 1, **Figure 1**) produced the strongest effect on enzyme activity, increasing initial velocities by ~1.8- to 2.2-fold of control levels. In this study, we used the atomic coordinates and a scoring grid of Site 1 to dock a library of 1217 FDA-approved compounds.²³ The coordinates of the 1217 compounds were docked into the hinge Site 1 (DOCK5.1, UCSF), and ranked by grid-based energy scores (van der Waals + electrostatic). **Figure 1** illustrates the binding pocket selected for high-throughput molecular docking and the positions of high-scoring small-molecule compounds. The functional effects of the top-scoring compounds were evaluated by measuring their effects on ACE2 maximal velocity and K_m .

Effects of FDA-approved drugs on ACE2 catalytic activity

The highest scoring 38 compounds were tested under saturating substrate conditions for their ability to modulate ACE2 enzyme activity *in vitro*. **Figure 2** demonstrates docking scores and initial velocities determined in the screening of 27 top-scoring compounds in the presence of 50 μM substrate. Ten

compounds producing the highest initial velocities for ACE2 were selected for further evaluation. Their docking scores and kinetic parameters are reported in **Table 1**; chemical structures are shown in **Table 2** (see also **Table 3**). The previously described compound XNT that increases ACE2 activity was used as a reference. **Figure 3** demonstrates kinetic curves for ACE2 in the presence of selected compounds and 25 to 125 μM substrate.

Two of the FDA-approved compounds (labetalol [LAB] and diminazene [DMZ]) were shown to increase maximal velocity at least twofold (21 ± 1.92 and $41 \pm 2.69 \text{ RFU}\cdot\text{s}^{-1}$, respectively) compared to ACE2 alone ($10 \pm 1.30 \text{ RFU}\cdot\text{s}^{-1}$), whereas incubation with aprindine, minithixen, and hydroxyzine (APR, CTX, and HXZ) resulted in 2.1- to 3.5-fold reduction in K_m . Despite the fact that many of the compounds demonstrated enhancing effects on either V_{\max} or K_m , only 3 of the selected 10 compounds had a statistically significant effect on overall enzyme efficiency (V_{\max}/K_m)—HXZ, CTX, and DMZ.

Interestingly, some of the known antihypertensive agents were identified in this screen. For example, labetalol (2-hydroxy-5-(1-hydroxy-2-((1-methyl-3-phenylpropyl) amino) ethyl) benzamide) is an antihypertensive drug marketed as Normodyne.²⁴ None of the compounds that significantly affect ACE2 enzyme efficiency were previously used for the treatment of hypertension or cardiovascular disease. Hydroxyzine is an antihistamine used in the treatment of allergies and hyperalgesia, minithixen is a dopamine receptor antagonist used as antipsychotic, and diminazene is an antiprotozoan chemotherapeutic used in veterinary medicine. More information on the clinical uses of selected compounds can be found in the **Supplemental Figures S1 and S2**.

The magnitude of the effect of these compounds has particular significance when compared with clinical effects of ACE inhibitors used for the treatment of hypertension, which typically inhibit enzyme activity to levels below 10%.^{25,26} The FDA-approved drug diminazene (benzenecarboximidamide, 4,4'-(1-triazene-1,3-diyl)bis-, dihydrochloride) identified in this study was shown to be the most effective in this group of compounds (**Table 1**), and we selected it for further evaluation.

EC_{50} and Lineweaver-Burk analysis

A Lineweaver-Burk (LB) plot was generated for five compounds that had the strongest effect on ACE2 (**Suppl. Fig. S1**). The plot suggests that both DMZ and XNT bind outside the active site and increase V_{\max} ~4-fold and K_m ~2-fold ($K_m_{\text{ACE2}} = 40 \pm 1.46 \mu\text{M}$, $K_m_{\text{XNT}} = 92 \pm 14.55 \mu\text{M}$, and $K_m_{\text{DMZ}} = 82 \pm 10.34 \mu\text{M}$), but the exact mechanism of enhancement by these compounds might be different. The LB plot demonstrating effects of DMZ on initial velocity of ACE2 in the presence of varying concentrations of the drug is shown in **Figure 4**.

Table 1. Kinetic Parameters of ACE2 in the Presence of Select Compounds

Name	Rank (Library)	Score ΔG° (kcal/mol)	K_m (μM)	V_{max} (RFU·s $^{-1}$)	V_{max}/K_m (RFU M $^{-1}$ s $^{-1}$ · 10 4)
ACE2 at 0.1 M NaCl pH 7.4	—	—	40 ± 1.5	10 ± 1.3	25 ± 1.3
NSC 354677 (XNT)	#3 (NCI)	-55.38	92 ± 14.5	36 ± 3.1	39 ± 3.2
NSC 290956 (ESP)	#4 (FDA)	-47.10	36 ± 2.2	6 ± 1.2	16 ± 1.2
NSC 357775 (DMZ)	#7 (FDA)	-46.53	82 ± 10.3	41 ± 2.7	50 ± 2.8
NSC 169188 (HXZ)	#10 (FDA)	-45.43	11 ± 1.0	4 ± 0.6	36 ± 0.6
NSC 169899 (CTX)	#18 (FDA)	-43.51	13 ± 0.8	6 ± 0.7	46 ± 0.7
NSC 134434 (HCT)	#20 (FDA)	-43.20	20 ± 1.1	6 ± 0.8	30 ± 0.8
NSC 293901 (FMB)	#21 (FDA)	-43.10	33 ± 1.3	9 ± 1.1	27 ± 1.1
NSC 289337 (TIA)	#30 (FDA)	-42.04	23 ± 1.1	7 ± 0.9	30 ± 0.9
NSC 284614 (APR)	#32 (FDA)	-41.96	19 ± 1.5	4 ± 0.8	21 ± 0.8
NSC 290312 (LAB)	#35 (FDA)	-40.52	60 ± 12	21 ± 1.9	35 ± 2.0

ACE2, angiotensin-converting enzyme 2; FDA, Food and Drug Administration; NCI, National Cancer Institute; RFU, relative fluorescent unit.

Titration of ACE2 with diminazene (0.01–1000 μM) results in a biphasic dose–response curve illustrated in **Figure 5**. At low concentrations, the enzyme is activated with an EC_{50} of 8.04 μM , whereas at high concentrations, it is partially inhibited with an IC_{50} of 200.01 μM . Remarkably, even after the partial inhibition, ACE2 initial velocity remains significantly higher than in the absence of the drug. Previously discovered XNT and resorcinolnaphthalene ($EC_{50} = 20 \pm 0.8$ and 20 ± 0.4 μM)¹³ produced only about 70% of the maximal increase in initial velocity of diminazene.

HPLC analysis of Ang II cleavage

It has been determined that vasoconstrictor peptide angiotensin II (Ang II) is a preferred natural substrate for ACE2.²⁷ Angiotensin 1–7 (Ang 1–7), generated by enzymatic cleavage of Ang II, is one of the main effectors responsible for beneficial effects of ACE2 on the cardiovascular system.²⁸ Classical photo- and fluorimetric methods could not provide accurate detection and quantification of Ang 1–7 formation, and therefore we used a liquid chromatography/mass spectrometry (LC-MS)–based

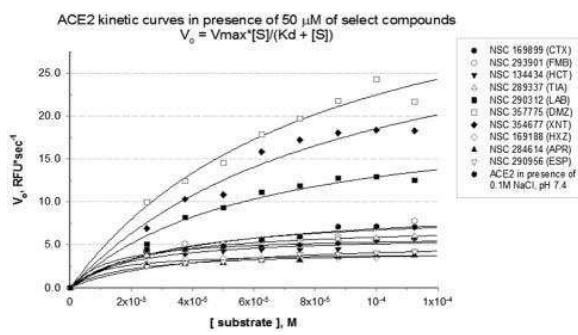


FIG. 3. Food and Drug Administration–approved drugs enhance angiotensin-converting enzyme 2 (ACE2) catalytic activity. ACE2 kinetic parameters were determined for 10 compounds that demonstrated highest initial velocities. Kinetic constants for ACE2 in the presence of the 50- μM compounds and 25 to 125 μM substrate were determined by nonlinear regression fit of experimental data into Michaelis-Menten equation $V_0 = \frac{V_{max} \cdot [S]}{K_m + [S]}$

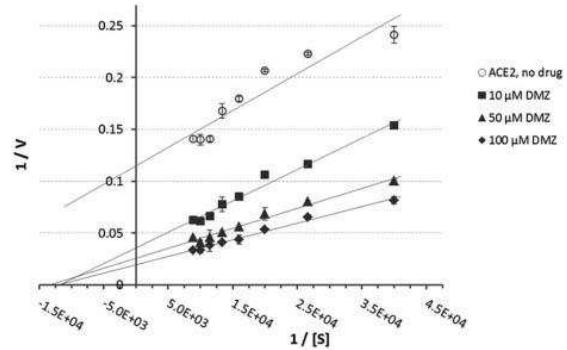


FIG. 4. Lineweaver-Burk double-reciprocal plot for angiotensin-converting enzyme 2 (ACE2) in the presence of 0 (○), 10 μM (■), 50 μM (▲), and 100 μM (◊) diminazene (DMZ). Despite the fact that even low concentrations of diminazene are sufficient to produce a boost in maximal velocity, overall enzyme efficiency responds more slowly to increasing concentrations of the compound and starts to approach 2.5-fold of the control levels only in presence of 100 μM drug.

Prediction of Off-Target Effects on ACE2

Table 2. Chemical Structures of Selected Food and Drug Administration–Approved Compounds

NSC Number	Chemical Name	Structure
NSC 354677 (XNT)	1-[[2-dimethylamino)ethyl] amino]-4-(hydroxymethyl)-7-[[[(4-methylphenyl) sulfonyl]oxy]-9H-xanthen-9-one	
NSC 290956 (ESP)	8-[3-(2-chlorophenothenothiazin-10-yl)propyl]-4-thia-1,8-diazaspiro[4.5]decan-2-one hydrochloride	
NSC 357775 (DMZ)	4-[2-(4-carbamimidoylphenyl) iminohydrazinyl]benzene-carboximidamide dihydrochloride	
NSC 169188 (HXZ)	2-[2-[4-[(4-chlorophenyl)-phenylmethyl] piperazin-1-yl]ethoxy]ethanol	
NSC 169899 (CTX)	(3Z)-3-(2-chlorothioxanthen-9-ylidene)-N,N-dimethylpropan-1-amine hydrochloride	
NSC 134434 (HCT)	1-(2-diethylaminoethylamino)-4-(hydroxymethyl)thioxanthen-9-one	
NSC 293901 (FMB)	N-[4-chloro-2-[[methyl-(2-morpholin-4-yl-2-oxoethyl)amino]methyl]phenyl] benzamide hydrochloride	
NSC 289337 (TIA)	5-chloro-3-[2-[4-(2-hydroxyethyl)piperazin-1-yl]-2-oxoethyl]-1, 3-benzothiazol-2-one hydrochloride	
NSC 284614 (APR)	N'-(2,3-dihydro-1H-inden-2-yl)-N,N-diethyl-N'-phenylpropane-1,3-diamine hydrochloride	
NSC 290312 (LAB)	2-hydroxy-5-[1-hydroxy-2-(4-phenylbutan-2-ylamino)ethyl]benzamide hydrochloride	

Table 3. Effect of Diminazene (DMZ) on Enzyme Efficiency

	V_{max}	K_m	V_{max}/K_m
10 μ M DMZ	32.75	1.00E-04	3.28·10 ⁵
50 μ M DMZ	41.00	8.23E-05	4.98·10 ⁵
100 μ M DMZ	56.62	9.76E-05	5.80·10 ⁵
ACE2	9.58	3.96E-05	2.42·10 ⁵

ACE2, angiotensin-converting enzyme 2.

assay system to directly analyze ACE2-catalyzed hydrolysis of Ang II and validate our kinetic assay data.

A 2-h incubation of 50 μ M Ang II with 10 nM ACE2 in the presence of 50 μ M DMZ resulted in a 1.5-fold increase in Ang (1–7) formation compared to that in the absence of the drug. MALDI-TOF spectrometry was used to determine peak identities and confirmed the generation of Ang (1–7) (899 m/z), which provides direct evidence for Ang II cleavage by ACE2. **Figure 6** shows HPLC chromatograms for ACE2 in the presence and absence of DMZ.

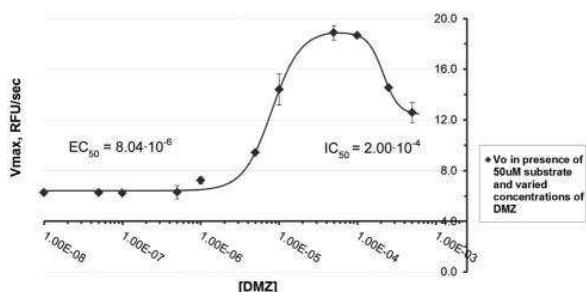


FIG. 5. Angiotensin-converting enzyme 2 (ACE2) dose–response curve for activation with diminazene (DMZ). In total, 10 nM ACE2 was incubated with 10 nM to 1 mM diminazene for 5 min at 37 °C. The reaction was initiated by addition of fluorogenic peptide substrate Mc-APK-(Dnp)-OH and monitored for 15 min. Diminazene enhances ACE2 activity in a dose-dependent manner with an EC₅₀ of 8.04 μM (concentration to achieve 50% increase in initial velocity). Titration results in a biphasic dose–response curve: at low concentrations of DMZ, the enzyme is activated, whereas at high concentrations, it is partially inhibited with an IC₅₀ of 200 μM.

DISCUSSION

We designed a study that focuses on a newly discovered enzyme that is a key regulator of hypertension because high blood pressure is a frequently observed and documented off-target effect. We targeted a specific structural pocket in the hinge-bending region of ACE2 (using the coordinates of the crystal structure of ACE2 in the open conformation) and found three FDA-approved compounds that significantly modulated ACE2 activity: hydroxyzine (HXZ), minithixen (CTX), and diminazene (DMZ). Hydroxyzine and minithixen act by increasing specificity for the synthetic substrate, which is reflected by reduction in K_m (K_m_{HXZ} = 11 ± 1.0 μM, K_m_{CTX} = 14 ± 0.8 μM vs K_m_{ACE2 alone} = 40 ± 1.5 μM). In contrast, diminazene and labetalol produce a large increase in V_{max}, which comes at a cost of loss in substrate specificity (K_m_{DMZ} = 82 ± 10.3 μM; **Table 1**). These data are consistent with the hypothesis that ACE2 conformational changes associated with substrate binding and/or product release may be rate limiting. This becomes particularly important in proteolytic cleavage of longer substrates, where fast turnover might be more difficult to achieve.

The effectiveness of FDA-approved diminazene as an ACE2 activator was confirmed by analysis of cleavage of the octapeptide angiotensin II, which is considered the main effector of the renin-angiotensin system (RAS) and the most physiologically significant natural substrate for ACE2. ACE2 EC₅₀ values for diminazene fall in the low micromolar range (8.0 μM) and demonstrate stronger response to this FDA-approved compound than to XNT, a non-FDA-approved drug-like small

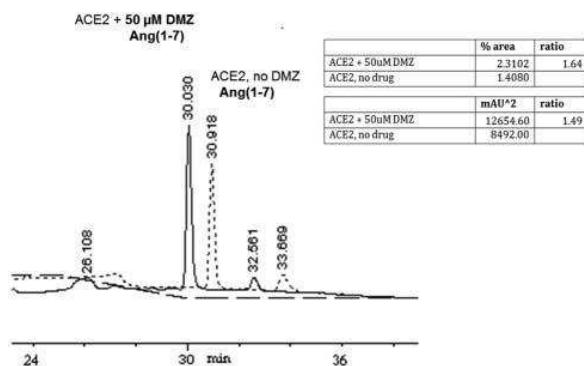


FIG. 6. Diminazene (DMZ) enhances cleavage of natural substrate angiotensin II (Ang II). High-performance liquid chromatography (HPLC) analysis of angiotensin II cleavage was performed by incubating 10 nM angiotensin-converting enzyme 2 (ACE2) with 50 μM Ang II in the presence of 50 μM DMZ and in the absence of the drug. Peptide products were separated by HPLC, and their identities were assigned by matrix-assisted laser desorption/ionization (MALDI). Mass spectrometry confirmed the peak for Ang (1–7) at 899 m/z. Two-hour incubation with DMZ resulted in a 1.5-fold increase in Ang (1–7) formation compared to that in the absence of the drug (as determined by integrating area under the curve [AUC]).

molecule shown to reduce high blood pressure in the in vivo model systems.^{13,14} Repurposing or repositioning is a strategy that takes advantage of the information available on drugs that have been approved for clinical use to enable their use in achieving therapeutic goals not intended originally. Because new relevant drug targets are constantly emerging, strategies to validate their targeting in humans are urgently needed.

This study has implications on the specificity of some known drugs. For example, Normodyne, a beta-blocker used to treat hypertension, enhances ACE2 activity in vitro. Effects observed in vivo may be due to the interaction between this drug and a combination of molecular targets, possibly including ACE2. Drugs that are useful in treating psychosis, such as minithixen, enhance ACE2 activity, suggesting that low blood pressure may be considered a possible side effect of this treatment due to the promiscuity of these agents. However, because minithixen is considered safe in humans, experiments may be designed to test its utility in treating hypertension. Similarly, the antibacterial and antifungal drugs diminazene and hydroxyzine, respectively, enhance ACE2 activity, suggesting that these compounds, or related analogs, may be useful in controlling blood pressure. These findings suggest that identification of off-target proteins that FDA-approved drugs modulate can be achieved by molecular docking. This strategy is both an informative way to illuminate possible undesired side effects and a rapid way to screen approved compounds for new clinical purposes.

REFERENCES

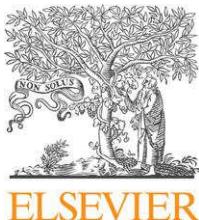
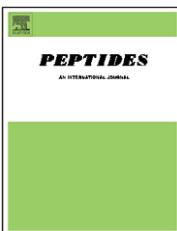
- Kuntz, I. D. Structure-Based Strategies for Drug Design and Discovery. *Science* **1992**, *257*, 1078–1082.
- Lindsley, C. W. The Akt/PKB Family of Protein Kinases: A Review of Small Molecule Inhibitors and Progress towards Target Validation: A 2009 Update. *Curr. Top. Med. Chem.* **2010**, *10*, 458–477.
- Li, H. F.; Chen, Y.; Rao, S. S.; Chen, X. M.; Liu, H. C.; Qin, J. H.; Tang, W. F.; Yue, W.; Zhou, X.; Lu, T. Recent Advances in the Research and Development of B-Raf Inhibitors. *Curr. Med. Chem.* **2010**, *17*, 1618–1634.
- Gao, Q.; Yang, L.; Zhu, Y. Pharmacophore Based Drug Design Approach as a Practical Process in Drug Discovery. *Curr. Comput. Aided Drug Des.* **2010**, *6*, 37–49.
- Villoutreix, B. O.; Eudes, R.; Miteva, M. A. Structure-Based Virtual Ligand Screening: Recent Success Stories. *Comb. Chem. High Throughput Screen.* **2009**, *12*, 1000–1016.
- Talele, T. T.; Khedkar, S. A.; Rigby, A. C. Successful Applications of Computer Aided Drug Discovery: Moving Drugs from Concept to the Clinic. *Curr. Top. Med. Chem.* **2010**, *10*, 127–141.
- Annanne, D. Improving Clinical Trials in the Critically Ill: Unique Challenge—Sepsis. *Crit. Care Med.* **2009**, *37*, S117–S128.
- Staessen, J. A.; Richart, T.; Wang, Z.; Thijss, L. Implications of Recently Published Trials of Blood Pressure–Lowering Drugs in Hypertensive or High-Risk Patients. *Hypertension* **2010**, *55*, 819–831.
- Verdecchia, P.; Gentile, G.; Angeli, F.; Mazzotta, G.; Mancia, G.; Reboldi, G. Influence of Blood Pressure Reduction on Composite Cardiovascular Endpoints in Clinical Trials. *J. Hypertens.* **2010**, *28*, 1356–1365.
- Bonomi, P. D. Implications of Key Trials in Advanced Nonsmall Cell Lung Cancer. *Cancer* **2010**, *116*, 1155–1164.
- Donoghue, M.; Hsieh, F.; Baronas, E.; Godbout, K.; Gosselin, M.; Stagliano, N.; Donovan, M.; Woolf, B.; Robison, K.; Jeyaseelan, R.; et al. A Novel Angiotensin-Converting Enzyme-Related Carboxypeptidase (ACE2) Converts Angiotensin I to Angiotensin 1–9. *Circ. Res.* **2000**, *87*, E1–E9.
- Tipnis, S. R.; Hooper, N. M.; Hyde, R.; Karran, E.; Christie, G.; Turner, A. J. A Human Homolog of Angiotensin-Converting Enzyme: Cloning and Functional Expression as a Captopril-Insensitive Carboxypeptidase. *J. Biol. Chem.* **2000**, *275*, 33238–33243.
- Hernandez Prada, J. A.; Ferreira, A. J.; Katovich, M. J.; Shenoy, V.; Qi, Y.; Santos, R. A.; Castellano, R. K.; Lampkins, A. J.; Gubala, V.; Ostrov, D. A.; et al. Structure-Based Identification of Small-Molecule Angiotensin-Converting Enzyme 2 Activators as Novel Antihypertensive Agents. *Hypertension* **2008**, *51*, 1312–1317.
- Ferreira, A. J.; Shenoy, V.; Yamazato, Y.; Sriramula, S.; Francis, J.; Yuan, L.; Castellano, R. K.; Ostrov, D. A.; Oh, S. P.; Katovich, M. J.; Raizada, M. K. Evidence for Angiotensin-Converting Enzyme 2 as a Therapeutic Target for the Prevention of Pulmonary Hypertension. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, 1048–1054.
- Towler, P.; Staker, B.; Prasad, S. G.; Menon, S.; Tang, J.; Parsons, T.; Ryan, D.; Fisher, M.; Williams, D.; Dales, N. A.; et al. ACE2 X-Ray Structures Reveal a Large Hinge-Bending Motion Important for Inhibitor Binding and Catalysis. *J. Biol. Chem.* **2004**, *279*, 17996–18007.
- Ferrara, P.; Gohlke, H.; Price, D. J.; Klebe, G.; Brooks, C. L. III. Assessing Scoring Functions for Protein-Ligand Interactions. *J. Med. Chem.* **2004**, *47*, 3032–3047.
- Gschwend, D. A.; Good, A. C.; Kuntz, I. D. Molecular Docking towards Drug Discovery. *J. Mol. Recognit.* **1996**, *9*, 175–186.
- Ewing, T. J.; Makino, S.; Skillman, A. G.; Kuntz, I. D. DOCK 4.0: Search Strategies for Automated Molecular Docking of Flexible Molecule Databases. *J. Comput. Aided Mol. Des.* **2001**, *15*, 411–428.
- Driscoll, J. S. The Preclinical New Drug Research Program of the National Cancer Institute. *Cancer Treat. Rep.* **1984**, *68*, 63–76.
- Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—A Visualization System for Exploratory Research and Analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612.
- Palmier, M. O.; Van Doren, S. R. Rapid Determination of Enzyme Kinetics from Fluorescence: Overcoming the Inner Filter Effect. *Anal. Biochem.* **2007**, *371*, 43–51.
- Elased, K. M.; Cunha, T. S.; Gurley, S. B.; Coffman, T. M.; Morris, M. New Mass Spectrometric Assay for Angiotensin-Converting Enzyme 2 Activity. *Hypertension* **2006**, *47*, 1010–1017.
- Williams-DeVane, C. R.; Wolf, M. A.; Richard, A. M. DSSTox Chemical-Index Files for Exposure-Related Experiments in ArrayExpress and Gene Expression Omnibus: Enabling Toxicogenomics Data Linkages. *Bioinformatics* **2009**, *25*, 692–694.
- Ahuja, K.; Charap, M. H. Management of Perioperative Hypertensive Urgencies with Parenteral Medications. *J. Hosp. Med.* **2010**, *5*, E11–E16.
- Jorde, U. P.; Ennezat, P. V.; Lisker, J.; Suryadevara, V.; Infeld, J.; Cukon, S.; Hammer, A.; Sonnenblick, E. H.; Le Jemtel, T. H. Maximally Recommended Doses of Angiotensin-Converting Enzyme (ACE) Inhibitors Do Not Completely Prevent ACE-Mediated Formation of Angiotensin II in Chronic Heart Failure. *Circulation* **2000**, *101*, 844–846.
- Hanon, S.; Vijayaraman, P.; Sonnenblick, E. H.; Le Jemtel, T. H. Persistent Formation of Angiotensin II Despite Treatment with Maximally Recommended Doses of Angiotensin Converting Enzyme Inhibitors in Patients with Chronic Heart Failure. *J. Renin Angiotensin Aldosterone Syst.* **2000**, *1*, 147–150.
- Vickers, C.; Hales, P.; Kaushik, V.; Dick, L.; Gavin, J.; Tang, J.; Godbout, K.; Parsons, T.; Baronas, E.; Hsieh, F.; et al. Hydrolysis of Biological Peptides by Human Angiotensin-Converting Enzyme-Related Carboxypeptidase. *J. Biol. Chem.* **2002**, *277*, 14838–14843.
- Schindler, C.; Bramlage, P.; Kirch, W.; Ferrario, C. M. Role of the Vasodilator Peptide Angiotensin-(1-7) in Cardiovascular Drug Therapy. *Vasc. Health Risk Manag.* **2007**, *3*, 125–137.
- Valerio, L. G.; Yang, C.; Arvidson, K. B.; Kruhlak, N. L. A Structural Feature-Based Computational Approach for Toxicology Predictions. *Expert Opin. Drug Metab. Toxicol.* **2010**, *6*, 505–518.
- Natesh, R.; Schwager, S. L.; Sturrock, E. D.; Acharya, K. R. Crystal Structure of the Human Angiotensin-Converting Enzyme-Lisinopril Complex. *Nature* **2003**, *421*, 551–554.
- Mores, A.; Matziari, M.; Beau, F.; Cuniasse, P.; Yiotakis, A.; Dive, V. Development of Potent and Selective Phosphinic Peptide Inhibitors of Angiotensin-Converting Enzyme 2. *J. Med. Chem.* **2008**, *51*, 2216–2226.

Address correspondence to:

David A. Ostrov

Department of Pathology, University of Florida
2033 Mowry Road, Gainesville, FL 32611

E-mail: ostroda@pathology.ufl.edu

available at www.sciencedirect.comjournal homepage: www.elsevier.com/locate/peptides

Study of angiotensin-(1–7) vasoactive peptide and its β -cyclodextrin inclusion complexes: Complete sequence-specific NMR assignments and structural studies

Ivana Lula ^a, Ângelo L. Denadai ^{a,d}, Jarbas M. Resende ^a, Frederico B. de Sousa ^a, Guilherme F. de Lima ^a, Dorila Pilo-Veloso ^a, Thomas Heine ^c, Hélio A. Duarte ^a, Robson A.S. Santos ^b, Rubén D. Sinisterra ^{a,*}

^a Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil

^b Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

^c Institut für Physikalische Chemie und Elektrochemie, TU Dresden, D-01062 Dresden, Germany

^d Centro Federal de Educação Tecnológica, CEFET, MG, Campus VII, Timóteo, Brazil

ARTICLE INFO

Article history:

Received 5 May 2007

Received in revised form

6 August 2007

Accepted 6 August 2007

Published on line 19 August 2007

Keywords:

Angiotensin-(1–7)

NMR studies and ROESY

Cyclodextrins

Inclusion compounds

QM/MM

DFTB and UFF simulation

ABSTRACT

We report the complete sequence-specific hydrogen NMR assignments of vasoactive peptide angiotensin-(1–7) (Ang-(1–7)). Assignments of the majority of the resonances were accomplished by COSY, TOCSY, and ROESY peak coordinates at 400 MHz and 600 MHz. Long-side-chain amino acid spin system identification was facilitated by long-range coherence transfer experiments (TOCSY). Problems with overlapped resonance signals were solved by analysis of heteronuclear 2D experiments (HSQC and HMBC). Nuclear Overhauser effects (NOE) results were used to probe peptide conformation. We show that the inclusion of the angiotensin-(1–7) tyrosine residue is favored in inclusion complexes with β -cyclodextrin. QM/MM simulations at the DFTB/UFF level confirm the experimental NMR findings and provide detailed structural information on these compounds in aqueous solution.

© 2007 Elsevier Inc. All rights reserved.

* Corresponding author. Tel.: +55 31 3499 5778; fax: +55 31 3499 5700.

E-mail address: sinisterra@ufmg.br (R.D. Sinisterra).

Abbreviations: Ang-(1–7), angiotensin-(1–7); Ang-II, angiotensin II; Ang-I, angiotensin I; ACE, angiotensin converting enzyme; BK, bradykinin; CD, cyclodextrin; β -CD, β -cyclodextrin; DFTB, density-functional based tight-binding; NMR, nuclear magnetic resonance; 2D, two-dimensional; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; TOCSY, total correlation spectroscopy; ITC, isothermal calorimetry titration; MM, molecular mechanics; RAS, renin–angiotensin system; ROESY, rotating frame Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; ITC, isothermal calorimetry titration; UFF, universal force field.

0196-9781/\$ – see front matter © 2007 Elsevier Inc. All rights reserved.

doi:10.1016/j.peptides.2007.08.011

1. Introduction

The endogenous heptapeptide angiotensin-(1–7), Ang-(1–7), (Fig. 1), is a biologically active member of the angiotensin peptides family. Evidence suggests that it has a great potential to treat cardiovascular diseases due to its activity in the renin–angiotensin system (RAS), the most important regulatory system for cardiovascular homeostasis [40,41].

Metabolic studies have contributed to establish the major enzymatic pathways for generation of biologically activity of angiotensin peptides. The circulating angiotensinogen is acting by renin released from the kidney forming the decapeptide angiotensin I (Ang-I); once formed, can be processed generating several biologically active products; including Ang-(1–7). Ang-(1–7) which can be also generated by the cleavage of the post-proline bond of angiotensin II (Ang-II) by ACE2 [41], is the most important metabolic product generated by a pathway where angiotensin converting enzyme (ACE) action is not involved [7,40].

Ang-(1–7) display actions which are often opposite of those described for Ang II and contributes to the regulation of blood pressure, in particular, to the regulation of blood flow [40]. Ang-(1–7) inhibits vascular smooth muscle cell growth, lowers blood pressure under conditions of high Ang II production and activity and, as it has been shown recently to interact with the G Protein Coupled Receptor Mas [41]. Ang-(1–7) can increase in plasma after ACE inhibition or AT₁ receptor blockade [7,40], which allows that the interaction of Ang-(1–7) with Mas and BK-related mechanisms promote beneficial cardiovascular effects [17,18,33,40]. It has been demonstrated that Ang-(1–7) acts inside the RAS as a contraregulatory peptide of this system. Acting at multiple points Ang-(1–7) decreases angiogenesis and cellular proliferation [17,18,33,40].

Considering a pharmaceutical formulation, many protein and peptide drugs may not be administrated orally because of their degradation by the stomach and the intestine digestive

enzymes. The colon is the superior organ for peptide absorption after oral ingestion; many studies indicate that colon-specific drug carriers should be used to deliver peptide drugs to that organ [39].

Various therapeutic systems used for targeting drugs to the colon are capable of reducing the dose to be administrated, and possibly the adverse effects associated with drug degradation. A variety of compounds, including polysaccharides, have been used for colon-specific drug delivery. Polysaccharides can be easily biochemically modified and are chemically stable, safe, nontoxic, hydrophilic and biodegradable; hence, they are excellent candidates as drug delivery systems. A large number of polysaccharides, such as cyclodextrins, have been tried for their potential as colon-specific drug carrier systems [39,44].

Cyclodextrins are cyclic oligosaccharides. They consist of six to eight glucopyranose units, which are linked through α-1, 4'-glucosidic bonds, resulting in toroidal molecules with polar outer surface and an apolar cavity. Its amphiphilic character makes the molecules soluble and also allows the formation of supramolecular inclusion complexes stabilized by non-covalent interactions with a variety of guest molecules. Cyclodextrins are neither hydrolyzed nor absorbed in the stomach or the small intestine. However, the colon microflora breaks them into small saccharides, which are absorbed in the large intestine. Cyclodextrins can recognize the size and shape of peptides and proteins, but these molecules are too hydrophilic and bulky to be wholly included into cyclodextrin cavity. However, peptide drugs can be formed presenting a hydrophobic side chains in their backbones, which may interact and form inclusion complexes with cyclodextrins [11,23,29,45,47,48].

Solution state nuclear magnetic resonance spectroscopy (NMR) is an important tool to determine the structures of peptides, proteins, and other molecules. Resonance assignments form the basis of analysis of peptide structure, and their

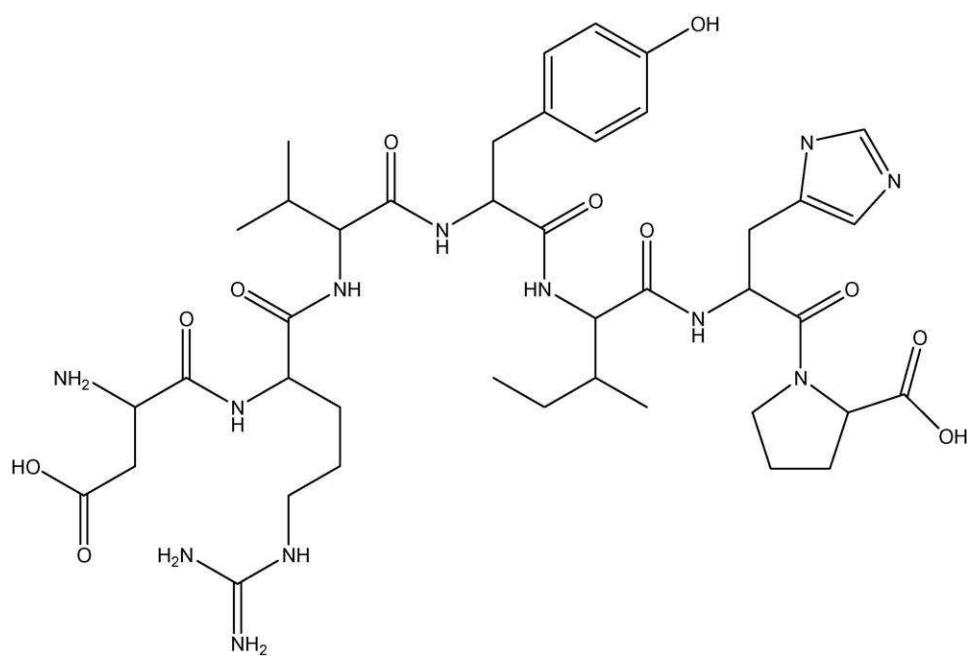


Fig. 1 – The chemical structure of angiotensin-(1–7), [Asp-Arg-Val-Tyr-Ile-His-Pro].

determination is the first step in the analysis of peptide solution structure. Rotating frame Overhauser enhancement spectroscopy (ROESY) experiments were used to probe interaction and conformational changes upon cyclodextrin interaction. Total correlation spectroscopy (TOCSY) experiments were used to identify individual resonances associated with each spin system, and to classify each identified spin system with respect to its amino acid residue [1,6]. Heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) were used to solve overlapping resonance problems and to assign ^{13}C NMR signals of the peptide [2,5,14,19]. The studies on Ang-(1-7) and its interaction with the β -cyclodextrin were completed with circular dichroism spectroscopy (CD), one of the most valuable techniques in the investigation of peptides structures in solution, and by isothermal calorimetry titration (ITC).

The purpose of this paper is to characterize the heptapeptide angiotensin-(1-7) with amino acid sequence Asp-Arg-Val-Tyr-Ile-His-Pro and its β -cyclodextrin inclusion compounds by employing different physical-chemical techniques and to attribute their NMR signals [1,2,5,6,14,19].

2. Materials and methods

2.1. Reagents

Angiotensin-(1-7) p.a. degree was obtained from BACHEM[®] Bioscience Inc., USA; and β -cyclodextrin p.a. degree from CERESTAR[®], USA. Deuterated water (D_2O), was provided by CIL—Cambridge Isotope Laboratories, Inc., and Milli-Q[®] water was used in sample preparation. All reagents were used as received.

2.2. Inclusion compounds preparation

The inclusion complex of Ang-(1-7) with β -cyclodextrin was prepared by freeze-drying method: solid-state Ang-(1-7) complex with cyclodextrin was prepared in 1:1 molar ratio (12 mM total concentration). The required amounts of Ang-(1-7) and β -cyclodextrin were accurately weighed and dispersed in Milli-Q[®] water and the solution was magnetically stirred at room temperature for 48 h before freeze-drying.

2.3. NMR experiments

NMR spectra were recorded on Bruker DRX400-AVANCE spectrometer operating at 400 MHz, Bruker DRX600-AVANCE and VARIAN INOVA-600AS spectrometer operating at 600 MHz equipped with a 5 mm inverse probe with z-gradient coil and a 5 mm gradient triple resonance (^1H - ^{13}C - ^{15}N / ^{31}P) probe, respectively. The 12 mmol NMR peptide sample was prepared in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ as a solvent, pH 2.90. One-dimensional ^{13}C NMR spectra were acquired on Bruker DRX400-AVANCE under standard conditions.

TOCSY contour maps (spin lock time of 80 ms, 120 ms and 220 ms) were acquired using MLEV-17 pulse sequence. NOESY contour maps were acquired using a mixing time of 230 ms, determinated after NOE buildup analysis (not shown); no spin diffusion was observed at this mixing time. The experiments

were recorded at 5 °C on Bruker DRX600 and VARIAN INOVA-600AS, 600 MHz spectrometers. Water suppression was achieved using the WATERGATE technique. Two-dimensional inverse hydrogen-detected heteronuclear shift correlation spectra were obtained by HSQC pulse sequence [$^1\text{J}(\text{C}, \text{H})$] and HMBC pulse sequence [^1J (C, H), $n = 2, 3$, and 4]. All experiments were used to confirm the peptide molecule and the inclusion compounds assignments. NMR data were processed and analyzed with the spectrometer's software and NMRPIPE & NMR View software, version 5.0.3 [5,12,24, 30,34,50,54].

2.4. NMR-based structure calculation

NMR-based structure calculation were performed using the Xplor-NIH software, version 2.14 [42]. Starting with an extended structure, 100 structures were generated using a simulated annealing protocol. This was followed by 18,000 steps of simulated annealing at 1000 K and a subsequent decrease in temperature in 6000 steps in the first slow-cool annealing stage. All the structures were analyzed with the program MOLMOL [26].

2.5. Circular dichroism spectroscopy

Circular dichroism spectra of Ang-(1-7) and its inclusion compounds solutions were recorded in duplicate on JASCO spectrophotometer Model J-720 at 298 K with a 0.1 cm path length cell cuvette. Wavelengths were measured from 190 to 320 nm, with a 0.5 nm step resolution, 100 nm min^{-1} sweep speed and 0.1 nm bandwidth. The spectra, an average of 4 scans, were processed with the JASCO software. Solvent spectral subtraction was performed. Secondary structure content was calculated from the CD spectra using PEPFIT software [20,38,51].

2.6. Isothermal calorimetry titration

Calorimetric titration was carried out in water at 298.15 K in duplicate on a VP-ITC microcalorimeter from Microcal. The ITC instrument was previously calibrated electrically and chemically [31,46]. The titration experiments consisted of 51 successive injections of Ang-(1-7) (100 mM) into the reaction cell charged with 1.5 mL of β -cyclodextrin solution (5 mM), with time intervals in 350 s. To eliminate diffusion effects of the material from the syringe to the calorimetric cell, the first injection was discarded and subsequently a constant volume of 5 μL was injected with injection time of 2 s. The results were analyzed by the equipment software Micro Cal Origin 5.0 for ITC, and the subtraction of blank experiment (dilution of Ang-(1-7) in water) [13,46].

2.7. Computations

All calculations have been performed with the experimental version of the deMon code, which is available free of charge for personal and academic use [27]. For QM/MM calculations, the QM part was treated with the DFTB method [35,43] including the second-order density correction scheme (self-consistent charge, SCC) [16], and the correction for London dispersion

(dispersion correction, DC) as implemented in deMon (DC-SCC-DFTB) [8,52]. The SCC-DFTB method has been thoroughly tested for biological molecules by Elstner and coworkers [53], and hybrid calculations involving SCC-DFTB for systems of biological interest have been reported earlier [4,15,21]. The molecular mechanics part employs Rappé's universal force field (UFF), with the partial charges taken from the TIP-3P interaction potential for water [25,37]. The chosen MM scheme yields a self-diffusion coefficient of water of $3.37 \times 10^{-5} \text{ cm}^2/\text{s}$ at ambient conditions, which is higher than in experiment, but still in closer agreement than TIP-3P-based force fields [32,36].

The employed QM/MM [49] subtraction scheme with electrostatic embedding restricts the force field to act on the solvent and on the solvent–solute interactions. To account for the latter ones, fixed charges are put to the β -cyclodextrin molecule. The MM part of the simulation is carried out within periodic boundary conditions (PBC). Here, the Coulomb interactions are calculated using the Ewald technique and van der Waals interactions are computed within the basis box of the super cell applying the minimum image convention. For Ang-(1–7), the simulation box is cubic with an adequate lattice vector length of 55.15 Å, including 5564 water molecules and the solute. For the Ang-(1–7)/ β -cyclodextrin inclusion compound, the simulation box is also cubic with lattice vector length of 61.0 Å, including 7381 water molecules and the solute. The solute is calculated as a gas-phase molecule, embedded in the charge distribution of the solvent, using DC-SCC-DFTB.

For the Born–Oppenheimer molecular dynamics simulations the following protocol was followed: All trajectories were carefully heated-up and finally equilibrated for 20 ps using the Berendsen thermostat [3] with a coupling parameter of $\tau = 0.1\text{--}1 \text{ ps}$. In the microcanonical NVE (constant number of particles, volume and energy, that is, no energy transfer either from or to the medium) production runs of 20 ps with 0.5 ps time steps were chosen. The total energy remained constant within 0.001 Hartree during the whole simulation without drift.

For structure and dynamics calculation, details on the calculation of radial functions and diffusion coefficients are given in the respective sections.

3. Results

3.1. Circular dichroism spectroscopy

The circular dichroism spectra of Ang-(1–7), recorded with and without β -cyclodextrin, are shown in Fig. 2. In aqueous solution the spectrum of Ang-(1–7) presents two negative bands at 190 nm and 218 nm ($-6986^\circ \text{ cm}^2 \text{ mol}^{-1}$ and $-2076^\circ \text{ cm}^2 \text{ mol}^{-1}$), and two positive bands at 205 nm and 233.7 nm ($426^\circ \text{ cm}^2 \text{ mol}^{-1}$ and $234^\circ \text{ cm}^2 \text{ mol}^{-1}$). Under the same conditions the spectrum of Ang-(1–7)/ β -cyclodextrin solutions shows only a little shift of the first positive band for 203 nm ($-892^\circ \text{ cm}^2 \text{ mol}^{-1}$) and a reduction in the intensities of all signals (Fig. 2).

3.2. Isothermal calorimetry titration

Isothermal titration calorimetry (ITC) was used to evaluate the thermodynamic parameters of the supramolecular interac-

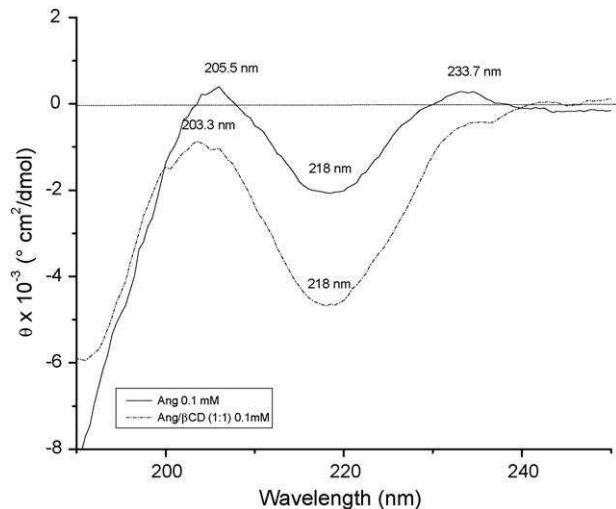


Fig. 2 – Circular dichroism spectra of Ang-(1–7) and Ang-(1–7)/ β -cyclodextrin solution.

tion between the species. Fig. 3 shows the Ang-(1–7) titration curve in pure water and in β -CD aqueous solution.

3.3. NMR spectroscopy

Modern NMR techniques based on gradient-pulsed fields were used in this study to determine and assign the structures of heptapeptide Ang-(1–7) and its inclusion compounds [1,2,5,6,14,19,30,34,50,54]. ^1H NMR was carried out employing the WATERGATE [34] technique and ^{13}C NMR resonance

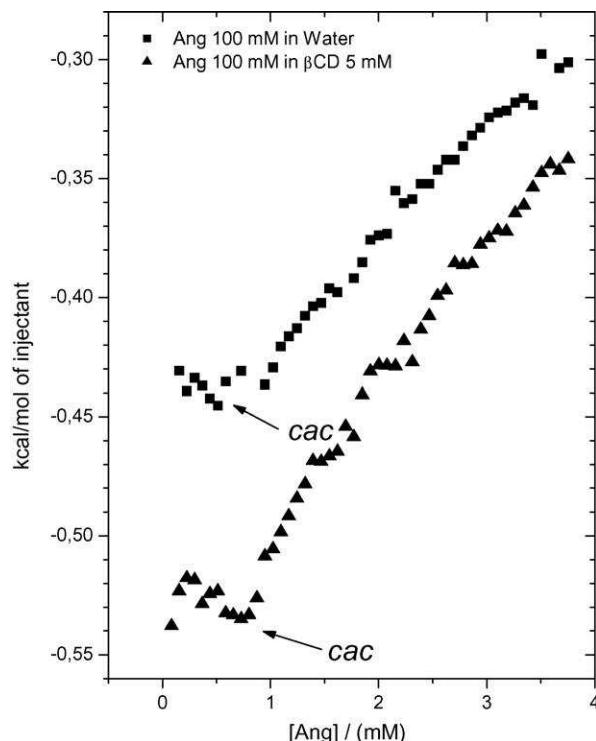


Fig. 3 – Calorimetric titration curve of Ang-(1–7)/ β -cyclodextrin solution.

Table 1 – ^1H NMR (600 MHz) spectral data for angiotensin-(1–7), 5 °C (10% $\text{D}_2\text{O}/\text{H}_2\text{O}$)

Residue	δ NH ($J_{\text{NH}-\text{H}\alpha}$)	δ α -H	δ β -H	δ others
Asp	–	4.02	2.61 and 2.72	–
Arg	8.46 (8.20)	4.03	1.41	γCH_2 δCH_2 =NH $\varepsilon\text{-NH}$
Val	8.08 (8.41)	3.76	1.64	γCH_3
Tyr	8.34 (7.26)	4.26	2.58	(2; 6) H (3; 5) H
Ile	7.88 (8.86)	3.72	1.38	γCH_2 γCH_3 δCH_3
His	8.43 (8.50)	4.48	2.87	(2) H (4) H
Pro	–	4.04	1.97	γCH_2 δCH_2
				0.55 and 0.59 6.74 6.41 0.74–0.83 and 1.04–1.11 0.47 0.46 8.30 7.02 1.68 3.17–3.25 and 3.47–3.55

assignments of the Angiotensin-(1–7) molecule were obtained by 2D shift-correlated NMR techniques. Hydrogen and carbon atom chemical shifts are summarized in **Tables 1 and 2**. The ^1H NMR chemical shift assignment of Ang-(1–7) was performed by sequential strategy as proposed by Wüthrich [50] (**Figs. 4 and 5**).

The exchange of amide hydrogens with deuterium can be monitored by the comparison of TOCSY contour maps recorded on 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ sample with data obtained from a freshly prepared sample in D_2O (**Fig. 6**). For the Ang-(1–7) in D_2O , the signals observed in the TOCSY contour map were designated to all five amide hydrogens which slowly exchange signals [9,10].

Table 3 lists the relative intensities of some of the NOE cross-peaks observed in the 2D-NOESY experiment. Approximately 113 NOE cross-peaks were assigned in the contour maps obtained at 5 °C, 600 MHz. Most of the NOEs were intraresidue (80 cross-peaks; $d = (i, i)$) and sequential (24 cross-peaks; $d = (i, i + 1)$); only nine cross-peaks were attributed to medium-range NOEs ($7d = (i, i + 2)$ cross-peaks and $2d = (i, i + 3)$) and the experiment did not show any long-range NOE cross-peaks ($d \geq (i, i + 4)$).

Analysis of the natural abundance of ^{13}C edited HSQC contour maps (recorded at 27 °C, 400 MHz and 5 °C, 600 MHz) also helped to resolve signal overlaps and amino acid spin systems recognition.

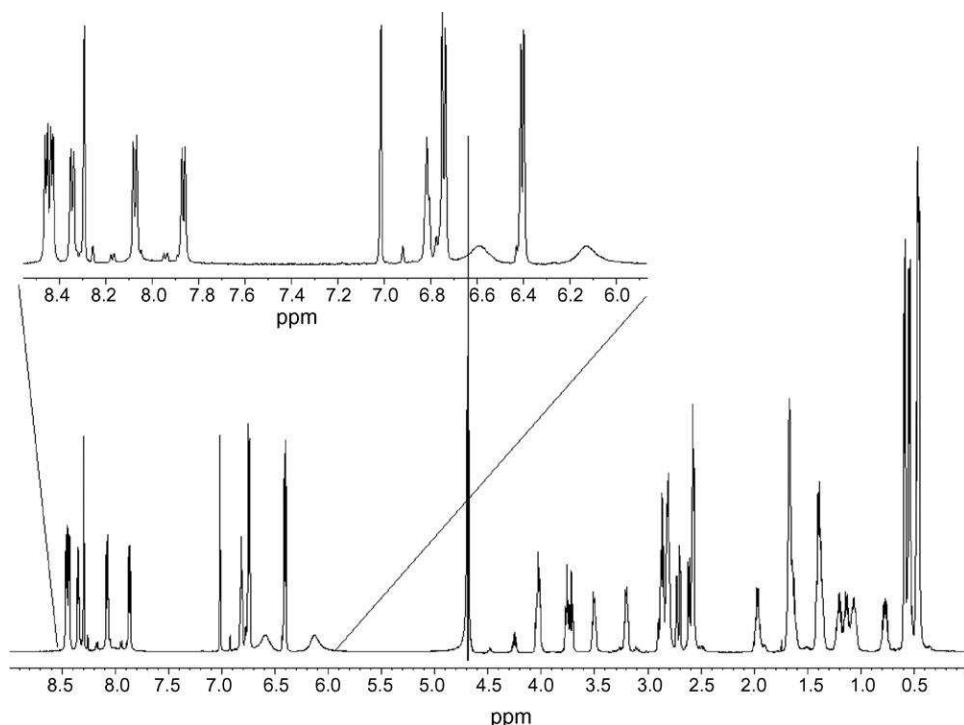


Fig. 4 – ^1H NMR spectra of Ang-(1–7) at 5 °C (WATERGATE, 600 MHz, 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$). Expansion of amidic and aromatic region (upper portion).

Table 2 – ^{13}C NMR (100 MHz) spectral data for angiotensin-(1–7), (D_2O)

Residue	δ α -CH	δ β -CH	δ others	
Asp	49.52	36.42	–	
Arg	54.77	24.55	γCH_2	28.21
			δCH_2	40.54
Val	59.26	30.38	γCH_3	18.34
			γCH_3	17.81
Tyr	53.47	35.27	(2 and 6) CH	30.55
			(3 and 5) CH	15.30
Ile	57.65	36.16	γCH_2	14.24
			γCH_3	9.79
			δCH_3	14.52
His	50.39	25.73	(2) CH	33.51
			(4) CH	30.51
Pro	59.96	29.00	γCH_2	24.55
			δCH_2	47.90

To know the host–guest interaction, the ^1H NMR employing WATERGATE and NOESY spectrums of Ang-(1–7)/ β -cyclodextrin complex in aqueous solution was obtained, Figs. 7 and 8. The NOESY contour shows intermolecular NOE correlation between hydrogens of Ang-(1–7) residue and the hydrogens of the β -cyclodextrin cavity (Fig. 8).

3.4. NMR-based structure calculation

The structure calculation of Ang-(1–7) was performed using distance restraints obtained from 113 cross-peaks of a NOESY contour map recorded at 600 MHz, 5 °C. NOE cross-peaks were integrated in the NOESY contour maps, and their volumes were converted to distances restraints, using calibration by Hyberts and collaborators [22] (Fig. 9a). The upper limits of the

distances restraints thus obtained were 2.8 Å, 3.4 Å and 5.0 Å (strong, medium, and weak NOEs, respectively).

3.5. Computer simulations

Fig. 9b shows the configurational space of the Ang-(1–7) during the simulation. During the simulation it was observed that the polypeptide backbone is relatively rigid and the residues flip around the equilibrium geometry. The radial distribution functions (RDFs) of the water surrounding the Tyr residue of Ang-(1–7) and the inclusion compound with β -CD are shown in Fig. 10. Fig. 11 shows the calculated structure of the Ang-(1–7)/ β -CD complex with eight water molecules closest to the Tyr center of mass.

4. Discussion

In the present manuscript, we describe the solution structure of the peptide angiotensin-(1–7) and its inclusion compound with β -cyclodextrin.

Peptides are known to be flexible in solution, as intermolecular long-range interactions are overwhelmed by solvent–solute interactions.

The circular dichroism spectroscopy results indicate a conformational equilibrium between the random coil and β -sheet conformations for the peptide and its inclusion compound solutions. The deconvolution of the spectral data shows that the secondary structure might be characterized as “general β -bend”, the result from a mixture of bend structures (Fig. 2).

The ITC curves for systems with very well-defined stoichiometries and high equilibrium constant normally show

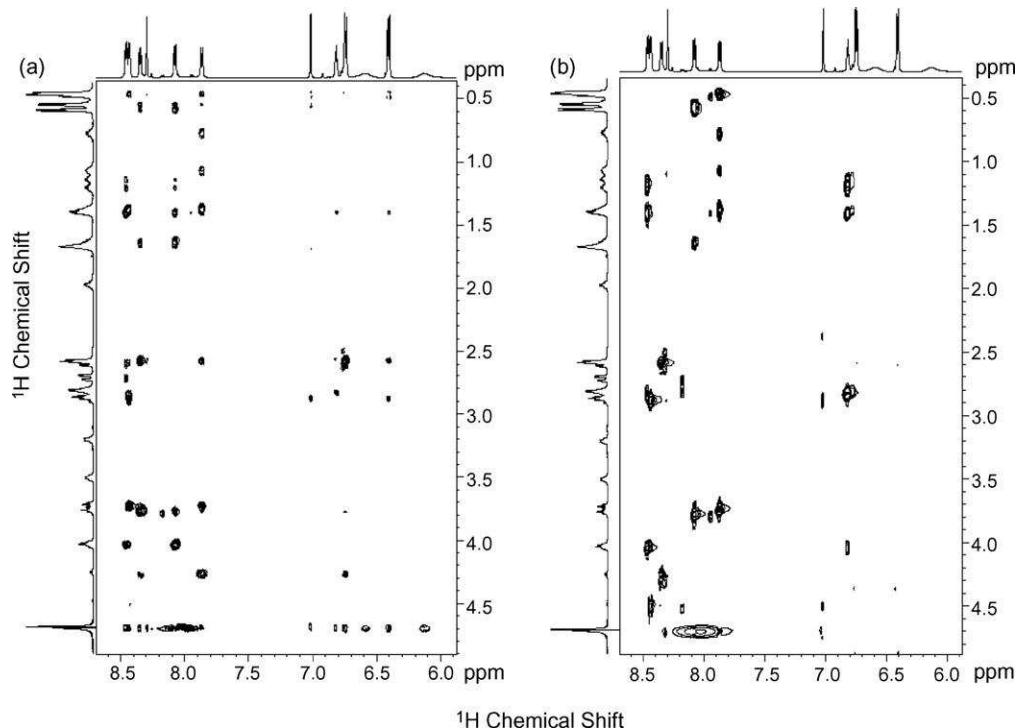


Fig. 5 – Amide and aromatic region of ^1H NMR spectra of Ang-(1–7) at 5 °C (600 MHz, 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$). (a) NOESY contour map of amidic region, mixing time of 230 ms. (b) TOCSY contour map of amidic region, mixing time of 80 ms.

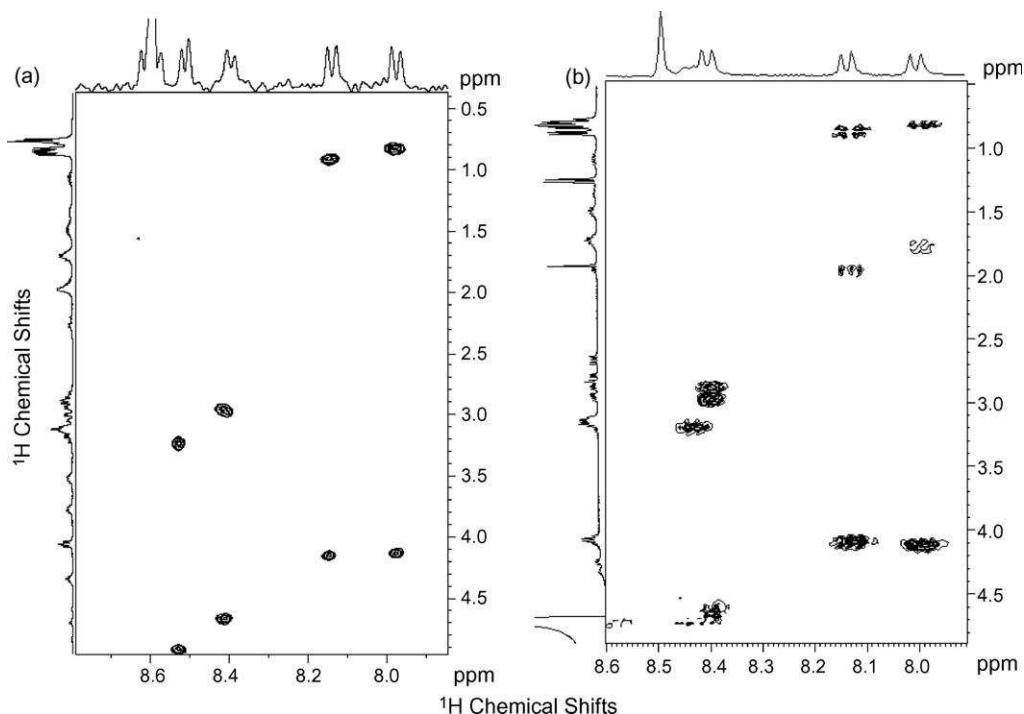


Fig. 6 – Amide region of ^1H NMR spectra of Ang-(1-7) at 27 °C (400 MHz). (a) TOCSY contour map of amidic region, D_2O . (b) TOCSY contour map of amidic region, 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$.

a sigmoidal profile whose plateau difference and inflection are the enthalpy and stoichiometry of the process, respectively [13,31,46] (Fig. 3).

The titration experiment of Ang-(1-7) in β -CD solution did not show a sigmoidal profile, indicating a very weak interaction between Ang-(1-7) and β -CD molecules. However, some important information may be obtained from this data. The titration curve of Ang-(1-7) in β -CD solution is more exothermic than that of the dilution of Ang-(1-7), showing that the interaction between the species is an exothermic process. This process may be attributed to the host-guest interactions, e.g. the formation of hydrogen bonds between -OH and -NH groups of Ang-(1-7) and $\text{C}_1\text{-O-C}_4$ groups of the cyclodextrin molecule, additional van der Waals interactions

with the aromatic groups of Ang-(1-7) and formation of hydrogen bonds between highly energetic water molecules released from cavity of β -CD with lattice water molecules [28].

Moreover, the dilution of Ang-(1-7) and titration experiment demonstrate that an inflection occurs at about 1 mM, probably due to the dissociation of angiotensin-(1-7) clusters, at the critical aggregation concentrations (cac).

NMR studies are suitable to monitor structural behavior in solution. The NMR results show which the peptide present a very well-defined structure in aqueous solution, confirmed by NMR structure calculation. The 1D NMR hydrogen spectra of the peptide in water shows mainly one set of chemical shifts for each amino acid residue, thus indicating conformational homogeneity. Only some minor

Table 3 – Selected NOEs in angiotensin-(1-7) in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$, 5 °C, 600 MHz^a

Residue	$\text{NH}_i\text{-NH}_{i+1}$	$\alpha_i\text{-NH}_i$	$\alpha_i\text{-NH}_{i+1}$	$\beta_i\text{-NH}_i/\text{NH}_{i+1}$	Others
Asp	–	–	–	NH Arg (m)	–
Arg	–	(s)	Val (s)	NH Val (m) NH Arg (m)	–
Val	–	(m)	Tyr (s)	NH Tyr (m) NH Val (s)	–
Tyr	Ile (w)	–	Ile (s)	NH Ile (m)	(3; 5) H Tyr- β His(w)
Ile	His (w)	(s)	His (s)	NH Ile (m)	–
	Tyr (w)	–	–	–	–
His	Ile (w)	(w)	–	NH His (s)	β His-(4) H His (m)
Pro	–	–	–	–	β Pro- α H Pro(w) β Pro- δ H _S Pro (w) β Pro- δ H _D Pro (w)

^a The letters w, m, and s correspond to weak, medium, and strong relative intensities of the NOE cross-peaks, respectively. S and D refer to the shield- and deshielded signals.

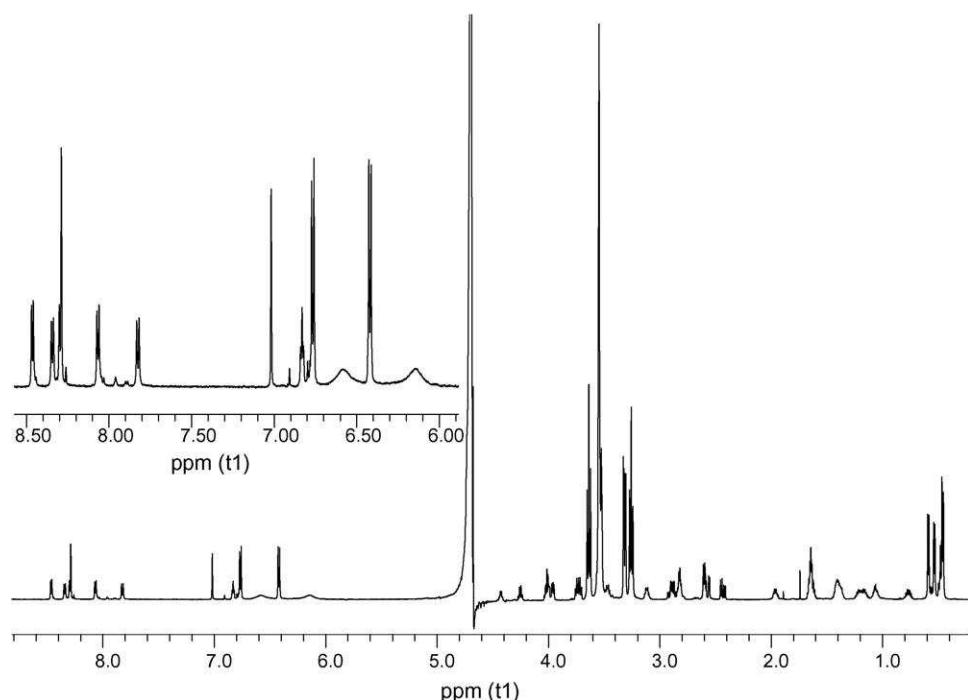


Fig. 7 – ^1H NMR spectra: Ang-(1-7)/ β -cyclodextrin complex at 5 °C, including expansion of amidic and aromatic region, (WATERGATE, 600 MHz, 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$).

additional peaks in the amidic region were observed (Fig. 4). Sequential connectivities could be observed through the analysis of TOCSY and NOESY contour maps (Fig. 5). Despite the good dispersion of amide hydrogen chemical shifts, values from δ 6.00 ppm to 8.60 ppm can be observed with

some signal overlaps attributable based on their connectivities to Arg and Val; Ile, Val and His signals.

The exchange NH- N^2H data enables the identification of potential regions of secondary structures in the peptide backbone. The NH resonance persisted for several months

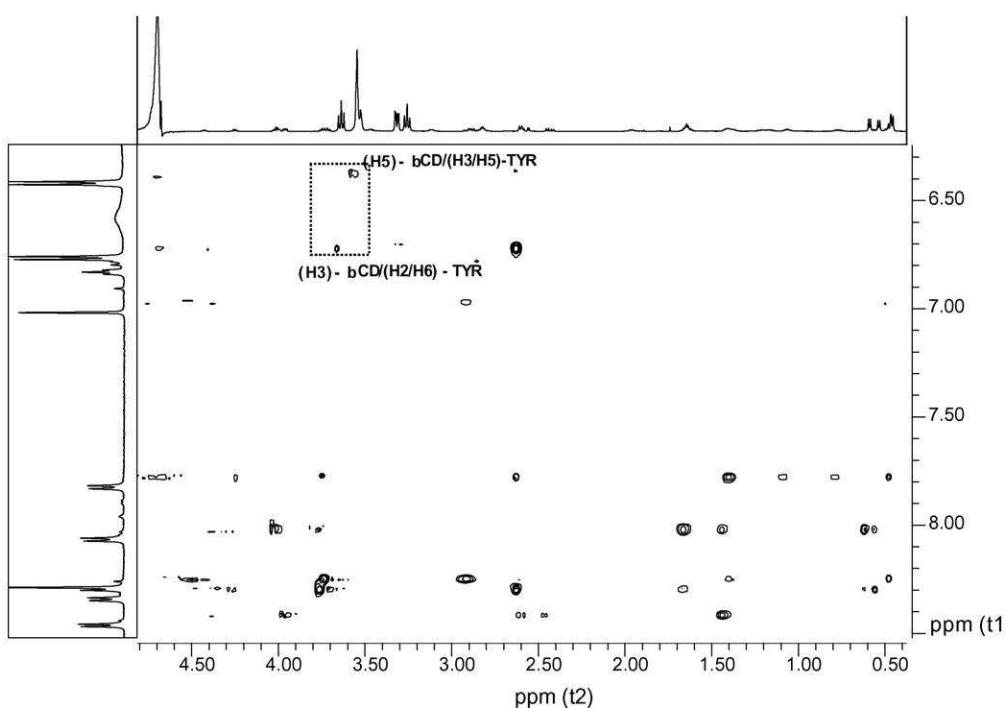


Fig. 8 – Expansion of amidic and aromatic region in NOESY contour map: Ang-(1-7)/ β -cyclodextrin complex at 5 °C, (600 MHz, $\text{D}_2\text{O}/\text{H}_2\text{O}$). For clarity, NOE correlations between Tyr residue and β -cyclodextrin hydrogens are indicated.

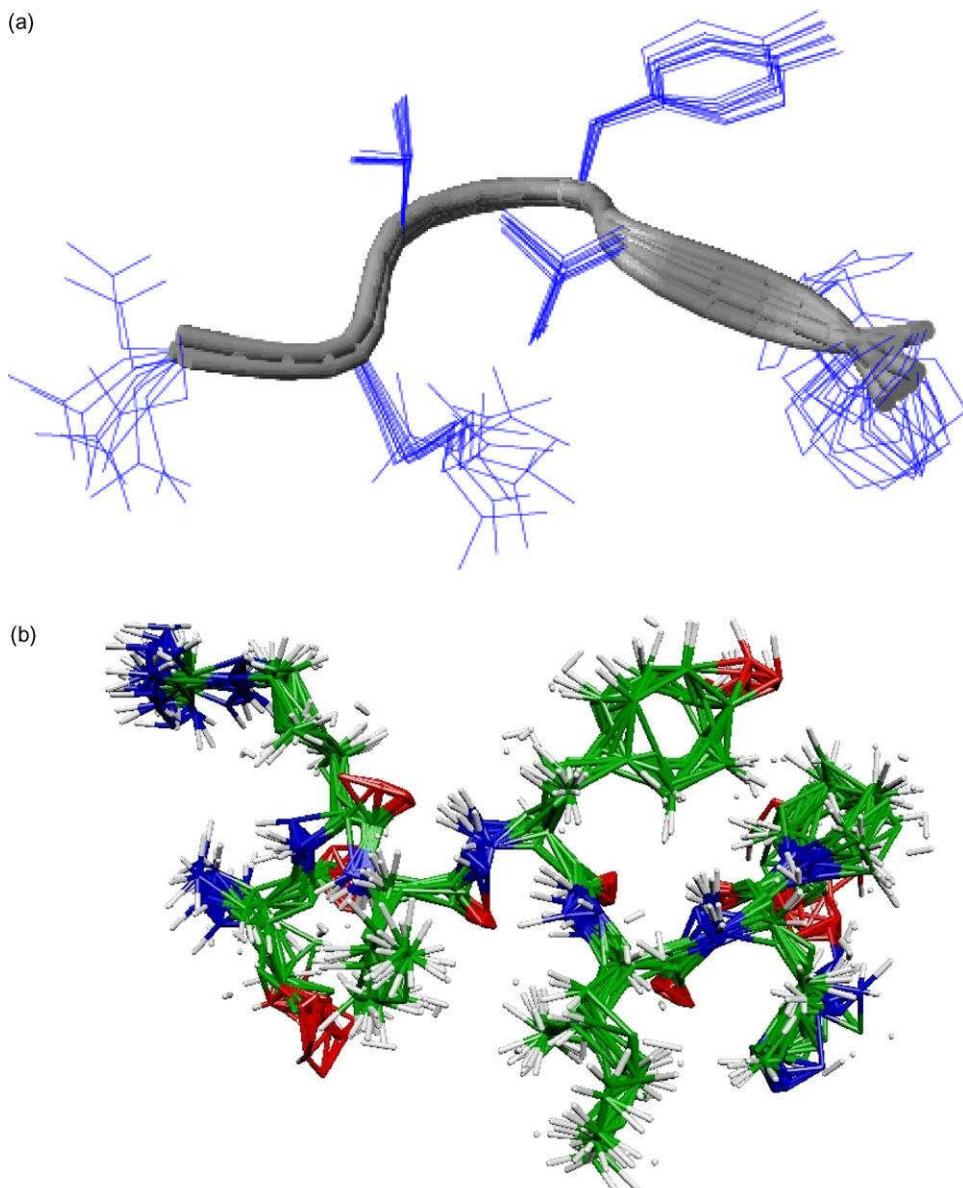


Fig. 9 – (a) NMR-based structure for angiotensin-(1-7) and (b) configurational space of angiotensin-(1-7) based on DFT/MM simulation (water molecules removed).

in angiotensin-(1-7) D_2O solution; this observation is consistent with the fact that these hydrogens take part in hydrogen bond which stabilizes the peptide secondary structure. The NMR-based structure calculation shows which the peptide backbone presents small flexibility of the Tyr and Arg residues in agreement with the computational simulations.

The free peptide showed very poor NOESY contour maps at 27 °C. Due to the high tumbling rate of the peptide NOEs with near zero intensity were generated, indicating that the peptide is very flexible in solution. The NOESY resolution increases for lower temperature (5 °C) and several cross-peaks may be observed, probably in function of the decreasing tumbling rates. The cross-peaks, listed in Table 3, involve correlations between peptide backbone protons and thus provide information about the peptide conformation. Some NH-NH and α H-NH

medium range NOE cross-peaks were observed, indicating the possibility of a predominant secondary structure. Additionally, the relative intensities of sequential α_i -NH $_{i+1}$ cross-peaks were greater than those of the corresponding intraresidue α_i -NH $_i$ cross-peaks, probably due to the eclipsed nature of the structure. The NOESY contour maps also showed weak-to-medium NOE cross-peaks between the α -hydrogens of His (δ 4.48) and the δ hydrogens of Pro (δ 3.17–3.25 and δ 3.47–3.55), which is only possible for a trans configuration between His-Pro bond, as previously mentioned by Cushman et al. [9].

Additionally, nuclear Overhauser effect measurement is one of the most important tools to prove the formation of the host-guest complex. NOESY technique is very useful to get insights on the supramolecular geometry of the complex.

The comparison between 1H NMR spectra of Ang-(1-7), Fig. 4, and 1H NMR spectra of Ang-(1-7)/ β -cyclodextrin

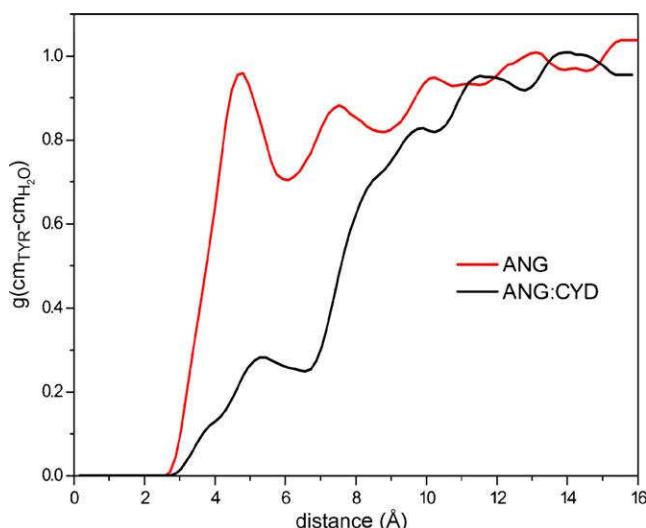


Fig. 10 – Radial distribution functions of the water around the Tyr residue in the angiotensin-(1-7).

complex in aqueous solution (Fig. 7) reveals some loss of resolution in the spectral lines of the NMR spectra, due to the complexation effects with the host molecule. The NOESY contour map of the Ang-(1-7)/β-cyclodextrin inclusion compound shows some differences in comparison with the free peptide spectrum. Only sequential and intraresidue cross-peaks were assigned in the contour maps. The experiment did not show any medium- or long-range NOE cross-peaks.

The NOESY contour map showed the intermolecular NOE correlation between hydrogens (3; 5) H (δ_H 6.41)/(2; 6) H (δ_H 6.74) of Tyr residue and the hydrogens 3 (δ_H 3.63) and 5 (δ_H 3.55) of the β-cyclodextrin cavity. These data suggest that the ring of the Tyr residue is situated deep in the torus cavity of cyclodextrin. A weak NOE effect between the NH protons and the methyl groups of Ile and Val residues of Ang-(1-7) with hydrogens 2 and 4 of the β-CD was also observed. Thus, the data suggests interactions between the residues of the Ang-(1-7) and the cyclodextrin molecules, with the formation of the Ang-(1-7)/β-cyclodextrin complex with 1:1 molar ratio (Fig. 8).

The NOEs intermolecular observed between the Ang-(1-7) and cyclodextrin hydrogens, in the Ang-(1-7)/β-cyclodextrin solution, as detected in the 2D-NOESY experiments, could only arise if an Ang-(1-7)/β-cyclodextrin complex had been formed. A structural modification for the peptide backbone for a fully extended and nonrigid conformation determinated by the NOE were attributed the host-guest interaction.

The structure obtained 10 lowest energy structures by NMR-based calculation showed RMSD (root mean square deviation) equal to 0.39 Å for backbone atoms and 1.18 Å for backbone and heavy atoms for all amino acid residues of Ang-(1-7). No NOE violations were observed. The 10 most stable structures show one bend between residues Val and Tyr, making a division in the peptide chain (Fig. 9a).

The structure calculation of Ang-(1-7)/β-cyclodextrin was performed using distance restraints obtained from cross-peaks in the NOESY contour map recorded at 600 MHz, 5 °C. The calculation does not show stable structures for the peptide in the inclusion compound, suggesting a fully

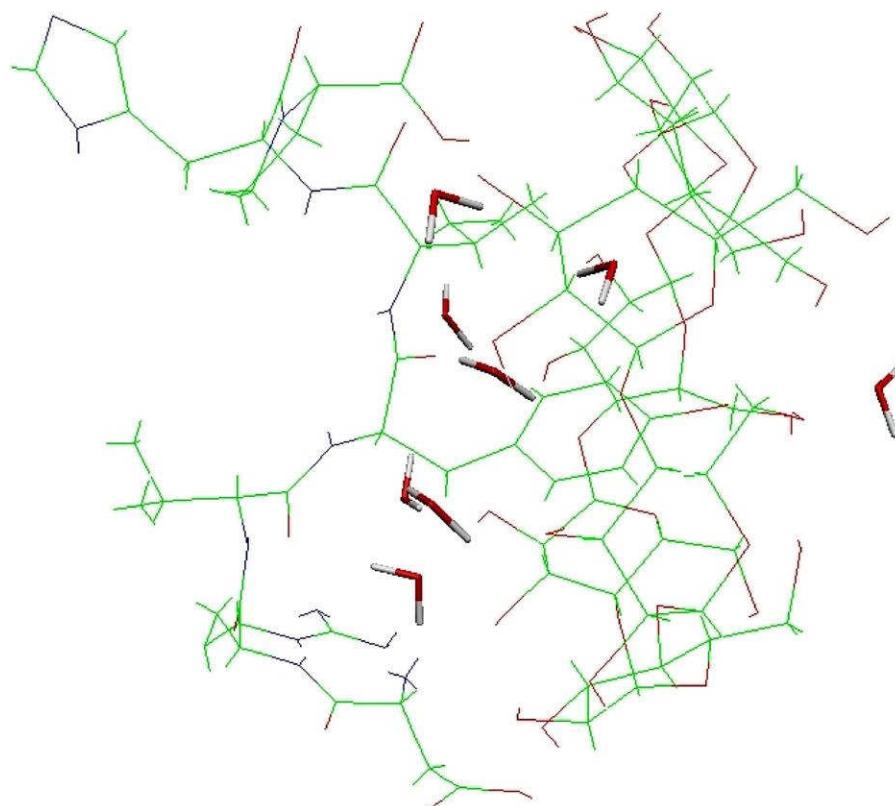


Fig. 11 – Snapshot of the Ang-(1-7)/β-CD complex with eight water molecules closest to the Tyr center of mass.

extended and nonrigid conformation for the peptide in the Ang-(1-7)/ β -cyclodextrin complex. This conformational modification can be attributed to the self-assembly angiotensin desegregation probably in function of the host-guest interaction.

The analysis of the computational simulation shows that the peptide dihedral angles ($N-C_{\alpha}-C-N$) have averages with large standard deviations of about 80 degrees (Fig. 9b). The smallest deviations are the ones assigned to the Tyr and Arg residues, which have standard deviations of $\sim 30^\circ$. The RDF results show that the first solvation shell around the Tyr is about 6 Å from its center of mass with about 19 water molecules. The RDF of the Ang-(1-7)/ β -CD shows clearly that the first solvation shell consists of only eight water molecules, which are mostly surrounding the rim of the β -CD and the hydroxyl group of the tyrosine residue (Fig. 11). The average distances between the Tyr H2/H3 and H6 of β -CD are about 3.55 ± 0.48 Å and those between Tyr H3/H5 and H5 of β -CD distances, the averages are about 2.74 ± 0.35 Å, corroborating with the NMR results which revealed NOE effect for those interactions.

5. Conclusions

Complexation of the angiotensin-(1-7) peptide with β -cyclodextrin was accomplished by a freeze-drying method with the inclusion of the Tyr residue in the cyclodextrin cavity. The structure was confirmed by NMR analysis and computer simulations. The former also allowed sequential assignment and the determination of the solution structure of the peptide. The NOE results obtained in two-dimensional ROESY and NOESY experiments gave further insight on the complex supramolecular geometry of the inclusion compounds and permitted structural calculations. Peptide Ang-(1-7) showed a well-defined β -sheet structure in solution, with a bend stabilized by interactions between residues 3 and 4 (Val and Tyr), and confirmed by circular dichroism and NMR data. The NOE results of Ang-(1-7) and its inclusion compound with β -cyclodextrin in aqueous solution shows which host-guest interactions break the peptide conformation.

DFTB/UFF simulations of the Ang-(1-7) and its β -cyclodextrin inclusion compound in aqueous solution were performed. The structural analyses are in good agreement with the experimental results, showing that β -cyclodextrin favors the inclusion of the Tyr residue.

6. Peptide accession numbers

The coordinates for the 10 refined, lowest energy structures have been deposited in the RCSB Protein Data Bank under ID: PDB ID (2JP8) and RCSB ID (RCSB100116).

Acknowledgment

This work was supported by the Brazilian research agencies Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do

Estado de Minas Gerais (FAPEMIG), and PRONEX-FAPEMIG (EDT 2403/03).

NMR facilities at 600 MHz were supported by CNRMN/UFRJ and LNLS-Brazilian Synchrotron Light Laboratory/MCT.

REFERENCES

- [1] Bax A, Davis DG. Practical aspects of two-dimensional transverse NOE spectroscopy. *J Magn Reson* 1985;63:207–13.
- [2] Bax A, Davis DG. MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy. *J Magn Res* 1985;65:355–60.
- [3] Berendsen HJC, Postma JPM, Vangunsteren WF, Dinola A, Haak JR. Molecular-dynamics with coupling to an external bath. *J Chem Phys* 1984;81:3684–90.
- [4] Bohr HG, Jalkanen KJ, Elstner M, Frimand K, Suhai S. A comparative study of MP2, B3LYP, RHF and SCC-DFTB force fields in predicting the vibrational spectra of N-acetyl-L-alanine-N'-methyl amide: VA and VCD spectra. *Chem Phys* 1999;246:13–36.
- [5] Braun S, Kalinowski H-O, Berger S. 150 and more basic NMR experiments—a practical course, 2nd ed., NY: WILEY-VCH; 1988.
- [6] Braunschweiler L, Schweiger A, Fauth JM, Ernst RR. Selective excitation in electron spin-echo modulation experiments. *J Magn Reson* 1985;64(1):160–6.
- [7] Campbell DJ, Kladis A, Duncan AM. Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension* 1994;23:439–49.
- [8] Cui Q, Elstner M, Kaxiras E, Frauenheim T, Karplus M. A QM/MM implementation of the self-consistent charge density functional tight binding (SCC-DFTB) method. *J Phys Chem B* 2001;105:569–85.
- [9] Cushman JA, Mishra PK, Bothner-By AA, Khosla MS. Conformations in solution of angiotensin II, and its 1-7 and 1-6 fragments. *Biopolymers* 1992;32:1163–71.
- [10] Davy SL, Osborne MJ, Moore GR. Determination of structure of oxidised *Desulfovibrio africanus* Ferrodoxin I by 1 H NMR spectroscopy and comparison of its solution structure with its crystal structure. *J Mol Biol* 1998;277:683–706.
- [11] Del Valle EM. Cyclodextrins and their uses: a review. *Process Biochem* 2004;39(9):1033–46.
- [12] Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–93.
- [13] Denadai AML, Santoro MM, Silva LH, Viana AT, Santos RAS, Sinisterra RD. Self-assembly characterization of β -cyclodextrin and hydrochlorothiazide system: NMR, phase solubility, ITC and QELS. *J Inclusion Phenom Macro Chem* 2006;55:41–9.
- [14] Derome AE. Modern NMR techniques for chemistry research, 1st ed., Pergamon Press: London; 1987.
- [15] Elstner M, Frauenheim T, Suhai S. An approximate DFT method for QM/MM simulations of biological structures and processes. *J Mol Struc Theochem* 2003;632:29–41.
- [16] Elstner M, Porezag D, Jungnickel G, Elsner J, Haugk M, Frauenheim T, et al. Self-consistent-charge density-functional tight-binding method for simulations of complex materials properties. *Phys Rev B* 1998;58:7260–8.
- [17] Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counter regulatory actions of angiotensin (1-7). *Hypertension* 1997;30:535–41.
- [18] Ferrario CM, Yier NS. Angiotensin-(1-7): a bioactive fragment of the renin-angiotensin system. *Regul Pept* 1998;78:13–8.

- [19] Gil VMS, Geraldes CFGC. Ressonância Magnética Nuclear—Fundamentos. Métodos e Aplicações, 1st ed., Lisboa: Fundação Calouste Gulbenkian; 1987.
- [20] Greenfield N, Fasman GD. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* 1969;8(10):4108–16.
- [21] Han WG, Elstner M, Jalkanen KJ, Frauenheim T, Suhai S. Hybrid SCC-DFTB/molecular mechanical studies of H-bonded systems and of N-acetyl-(L-Ala)(n) N'-methylamide helices in water solution. *Int J Quantum Chem* 2000;78:459–79.
- [22] Hyberts SG, Goldberg MS, Havel TF, Wagner G. The solution structure of eglin c based on measurements of many NOEs and coupling constants and its comparison with X-ray structures. *Protein Sci* 1992;1:736–51.
- [23] Irie T, Uekama K. Cyclodextrins in peptide and protein delivery. *Adv Drug Deliv Rev* 1999;36:101–23.
- [24] Johnson BA, Blevins RA. NMR View: a computer program for the visualization and analysis of NMR data. *J Biomol NMR* 1994;4:603–14.
- [25] Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys* 1983;79:926–35.
- [26] Koradi R, Billeter M, Wüthrich K. MOLMOL: a program for display and analysis macromolecular structures. *J Mol Graph* 1996;14:51–5.
- [27] Köster AM, Flores R, Geudtner G, Goursot A, Heine T, Patchkovskii S, Reveles JU, Vela A, Salahub DR deMon NRC: Canada; 2004.
- [28] Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J Pharm Sci* 1996;85(10):1017–25.
- [29] Loftsson T. Cyclodextrins in pharmaceutical formulations: the effects of polymers on their complexation efficacy and drug availability. Nordic Industrial Fund—a report; 1988.
- [30] Markley JL, Bax A, Arata Y, Hilbers CW, Kaptein R, Sykes BD, et al. Recommendations for the presentation of NMR structures of proteins and nucleic acids. *J Mol Biol* 1998;280:933–52.
- [31] MicroCal. ITC Data Analysis In origin®. In: MicroCal, editor. The calorimetry experts. 5.0 ed. MicroCal; 1998, <http://www.microcalorimetry.com>.
- [32] Mills R. Self-diffusion in normal and heavy-water in range 1–45 degrees. *J Phys Chem US* 1973;77:685–8.
- [33] Oliveira MA, Fortes ZB, Santos RAS, Kosla MC, De Carvalho MHC. Synergistic effect of angiotensin-(1–7) on bradykinin arteriolar dilation in vivo. *Peptides* 1999;20:1195–201.
- [34] Piotto M, Saudek V, Sklenár V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solution. *J Biomol NMR* 1992;2:661–5.
- [35] Porezag D, Frauenheim T, Kohler T, Seifert G, Kaschner R. Construction of tight-binding-like potentials on the basis of density-functional theory—application to carbon. *Phys Rev B* 1995;51:12947–5.
- [36] Price DJ, Brooks CL. A modified TIP3P water potential for simulation with Ewald summation. *J Chem Phys* 2004;121:10096–103.
- [37] Rappe AK, Casewit CJ, Colwell KS, Goddard WA, Skiff WM. Uff, a full periodic-table force-field for molecular mechanics and molecular-dynamics simulations. *J Am Chem Soc* 1992;114:10024–35.
- [38] Reed J, Reed TA. A set of constructed type spectra for the practical estimation of peptide secondary structure from circular dichroism. *Anal Biochem* 1997;254:36–40.
- [39] Rubstein A, Tirosh B, Baluom M, Nassar T, David A, Radai R, et al. The rationale of peptide drug delivery to the colon and the potential of polymeric carriers as effective tools. *J Control Release* 1997;46:59–73.
- [40] Santos RAS, Ferreira AJ, Pinheiro SV, Sampaio WO, Touyz R, Campagnole-Santos MJ. Angiotensin-(1–7) and its receptor as a potential targets for new cardiovascular drugs. *Expert Opin Investig Drugs* 2005;14:1019–31.
- [41] Santos RAS, Simões e Silva AC, Maric C, Silva DM, Machado RP, Buhr I, et al. Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 2003;100:8258–63.
- [42] Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. The Xplor-NIH NMR molecular structure determination package. *J Magn Reson* 2003;160:66–74.
- [43] Seifert G, Porezag D, Frauenheim T. Calculations of molecules, clusters, and solids with a simplified LCAO-DFT-LDA scheme. *Int J Quantum Chem* 1996;58:185–92.
- [44] Sinha VR, Kumria R. Polysaccharides in colon-specific drug delivery. *Int J Pharm* 2001;224:19–38.
- [45] Thompson DO. Cyclodextrins—enabling excipients: their present and future use in pharmaceuticals. *Crit rev Ther Drug Carrier Syst* 1997;14(1):1–104.
- [46] Turnbull WB, Daranas AH. On the value of c: can low affinity systems be studied by isothermal titration calorimetry? *J Am Chem Soc* 2003;125:14859–66.
- [47] Uekama K. Recent aspects of pharmaceutical application of cyclodextrins. *J Inclusion Phenom Macro Chem* 2002;44:3–7.
- [48] Uekama K, Hirayama F, Irie T. Cyclodextrin drug carrier system. *Chem Rev* 1998;98(5):2045–76.
- [49] Warshel A, Levitt M. Theoretical studies of enzymic reactions—dielectric, electrostatic and steric stabilization of carbonium-ion in reaction of lysozyme. *J Mol Biol* 1976;103:227–49.
- [50] Wüthrich K. NMR of protein and nucleic acids, 1st ed., NY: John Wiley & Sons, Inc.; 1986.
- [51] www.newark.rutgers.edu/chemistry/grad/chem585/lecture1.html.
- [52] Zhechkov L, Heine T, Patchkovskii S, Seifert G, Duarte HA. An efficient a posteriori treatment for dispersion interaction in density-functional-based tight binding. *J Chem Theory Comput* 2005;1:841–7.
- [53] Zhou HY, Tajkhorshid E, Frauenheim T, Suhai S, Elstner M. Performance of the AM1, PM3, and SCC-DFTB methods in the study of conjugated Schiff base molecules. *Chem Phys* 2002;277:91–103.
- [54] Zimmerman DE, Montelione G. Automated analysis of nuclear magnetic resonance assignments for proteins. *Curr Opin Struct Biol* 1995;5:664–73.



ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

The International Journal of Biochemistry & Cell Biology 38 (2006) 752–765

IJBCB

www.elsevier.com/locate/biocel

Review

Angiotensin and diabetic retinopathy

Jennifer L. Wilkinson-Berka *

Department of Physiology, The University of Melbourne, Grattan St., Parkville, Vic. 3010, Australia

Received 25 April 2005; received in revised form 25 July 2005; accepted 10 August 2005

Available online 1 September 2005

Abstract

Diabetic retinopathy develops in patients with both type 1 and type 2 diabetes and is the major cause of vision loss and blindness in the working population. In diabetes, damage to the retina occurs in the vasculature, neurons and glia resulting in pathological angiogenesis, vascular leakage and a loss in retinal function. The renin–angiotensin system is a causative factor in diabetic microvascular complications inducing a variety of tissue responses including vasoconstriction, inflammation, oxidative stress, cell hypertrophy and proliferation, angiogenesis and fibrosis. All components of the renin–angiotensin system including the angiotensin type 1 and angiotensin type 2 receptors have been identified in the retina of humans and rodents. There is evidence from both clinical and experimental models of diabetic retinopathy and hypoxic-induced retinal angiogenesis that the renin–angiotensin system is up-regulated. In these situations, retinal dysfunction has been linked to angiotensin-mediated induction of growth factors including vascular endothelial growth factor, platelet-derived growth factor and connective tissue growth factor. Evidence to date indicates that blockade of the renin–angiotensin system can confer retinoprotection in experimental models of diabetic retinopathy and ischemic retinopathy. This review examines the role of the renin–angiotensin system in diabetic retinopathy and the potential of its blockade as a treatment strategy for this vision-threatening disease.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Angiotensin; Renin; Retina; Diabetes; Ren-2 rat

Contents

1. Pathogenesis of diabetic retinopathy	753
2. The renin–angiotensin system (RAS)	754
3. The circulating RAS and diabetic retinopathy	754
4. The cellular location of the retinal renin–angiotensin system	755
5. Angiotensin and retinal vascular pathology	756
5.1. Pericytes	756
5.2. Angiogenesis	757

* Tel.: +61 3 83445849; fax: +61 3 83445818.

E-mail address: jlaberka@unimelb.edu.au.

6. Angiotensin 1–7 and ACE2.....	758
7. The streptozotocin diabetic transgenic (mRen-2)27 rat.....	759
8. Hypertension.....	759
9. Clinical studies.....	760
10. Summary.....	761
Acknowledgements.....	761
References.....	761

1. Pathogenesis of diabetic retinopathy

Vascular abnormalities are present in all patients who have had type 1 diabetes for 20 years and in approximately 80% of patients with type 2 diabetes for this time period (Frank, 2004). Vision loss in diabetic retinopathy develops by slow and progressive alterations to the retinal microvasculature (pericytes, endothelial cells) leading to breakdown of the blood–retinal barrier, pathological angiogenesis and scarring. Based on the extent of vascular abnormalities, diabetic retinopathy can be broadly categorised into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) (Klein, Klein, Moss, & Cruickshanks, 1998; Klein et al., 2004) (Fig. 1). The vascular lesions that develop in these stages will be briefly summarised.

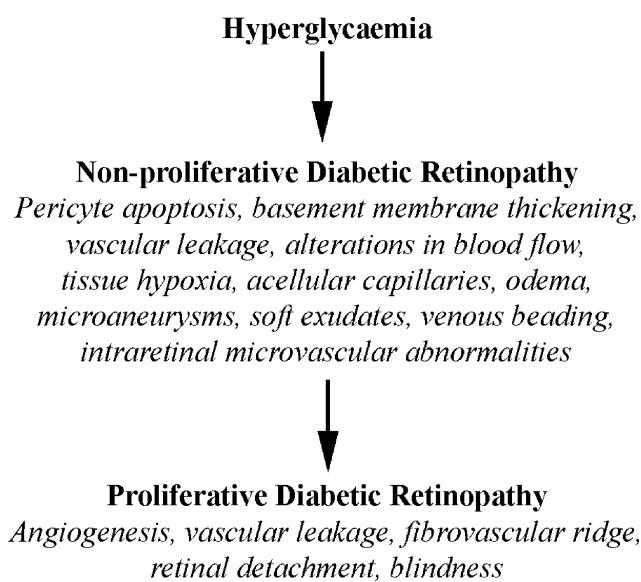


Fig. 1. A summary of the events that lead to vascular pathology in diabetic retinopathy. Hyperglycaemia initiates pericyte and endothelial cell injury that over time results in tissue hypoxia, vascular leakage and pathological angiogenesis.

In NPDR, hyperglycaemia induces thickening of capillary basement membrane, apoptosis or ‘drop-out’ of pericytes, microaneurysms and vascular leakage. Blockade of retinal capillaries causes localised hypoxia, which increases the production of angiogenic growth factors. In some microvessels, endothelial cells become apoptotic resulting in acellular capillaries (devoid of both pericytes and endothelial cells), capillary closure and areas of retinal non-perfusion. Adherent leukocytes may also contribute to the lesion by causing retinal capillary occlusion (Joussen et al., 2004). Multiple haemorrhages, soft exudates, cotton wool spots, intraretinal microvascular abnormalities and venous beading and loops develop. Increased areas of tissue non-perfusion stimulate the production of angiogenic factors leading to the proliferation of vessels, which is the hallmark feature of PDR. Retinal angiogenesis can be accompanied by fibrosis resulting in a fibrovascular ridge, which extends into the vitreous cavity or on the surface of the retina. Contraction of the fibrovascular ridge causes retinal detachment and vision loss and blindness (Watkins, 2003). Vision is also threatened by diabetic macula oedema, which occurs following breakdown of the blood–retina barrier (Klein, Klein, & Moss, 1992; Klein et al., 1998, 2004). In the late stages of diabetic retinopathy, angiogenesis and fibrosis can develop in the iris, leading to rubeosis iridis. This pathology affects the outflow of aqueous humor to result in neovascular glaucoma and an elevation in intraocular pressure, which compromises vision. The current treatment for diabetic retinopathy is laser photocoagulation, a procedure that destroys angiogenic vessels and the surrounding hypoxic tissue (Aiello, 2003). Although beneficial, laser photocoagulation can destroy healthy retina, and the disease continues despite intensive treatment. Currently, less invasive therapies are being investigated, with a particular focus on the inhibition of injurious growth factor systems. This review

will consider the possible benefits of RAS blockade.

2. The renin–angiotensin system (RAS)

The renin–angiotensin system is an enzymatic cascade in which angiotensinogen is the sole precursor of the angiotensin peptides. The cascade begins with the conversion of the inactive form of renin, prorenin, to active renin. This enzyme cleaves renin substrate (angiotensinogen) to generate angiotensin I (ANG I) (Fig. 2). Angiotensin II (ANG II) can be liberated from ANG I by angiotensin-converting enzyme (ACE) or serine proteases. ACE2 is a recently discovered homologue of ACE which cleaves ANG 1–7 from ANG II (Burrell, Johnston, Tikellis, & Cooper, 2004; Ye et al., 2004) (Fig. 2). Its functions are not fully defined; however, there is evidence that it may negatively regulate the RAS (Burrell et al., 2004; Ye et al., 2004). ANG II is the main effector peptide

of the RAS, acting primarily on two receptors, the angiotensin type I (AT1) and angiotensin type 2 (AT2) receptors (Chung, Kuhl, Stoll, & Unger, 1998).

The AT1 receptor elicits most of the known actions of ANG II (Aguilera & Kiss, 1996; Culman et al., 1995; Ito et al., 1995). It is a seven-transmembrane-domain G-protein-coupled receptor that is widely distributed in tissues including the vasculature, heart, brain, adrenals, kidneys, prostate and eye (Allen, Yamada, & Mendelsohn, 1990; Fabiani et al., 2003; Sarlos et al., 2003; Song, Allen, Paxinos, & Mendelsohn, 1992; Zhuo, Alcorn, McCausland, & Mendelsohn, 1994). Via the AT1 receptor, ANG II influences vasoconstriction, electrolyte homeostasis, modulation of drinking behavior and stimulation of pituitary hormone release (Aguilera & Kiss, 1996; Culman et al., 1995; Ito et al., 1995). ANG II is also a growth factor, promoting differentiation, apoptosis and the deposition of extracellular matrix (Kato et al., 1991; Otani, Takagi, Oh, Koyama, & Honda, 2001; Otani, Takagi, Suzuma, & Honda, 1998; Suzuki et al., 2003; Tamura et al., 1998). These roles for ANG II make it an important mediator of pathologies such as cardiac hypertrophy, myocardial infarction, atherosclerosis, kidney disease and cancer (Gilbert, Krum, Wilkinson-Berka, & Kelly, 2003; Kim & Iwao, 2000; Miyajima et al., 2002).

The AT2 receptor is a seven-transmembrane-domain G-coupled protein receptor whose signalling pathways involve: activation of protein phosphatases and protein dephosphorylation, regulation of the nitric oxide (NO)–cGMP system, stimulation of phospholipase A2 and release of arachidonic acid and sphingolipid-derived ceramide (Touyz & Berry, 2002). The AT2 receptor may oppose the actions of the AT1 receptor by inducing vasodilation and apoptosis (Chung et al., 1998). The overexpression of the AT2 receptor in fetal tissues and disease has suggested that in some situations the AT2 receptor has similar actions to the AT1 receptor, promoting cell growth and angiogenesis (Cao et al., 2000; Chung et al., 1998; Sarlos et al., 2003).

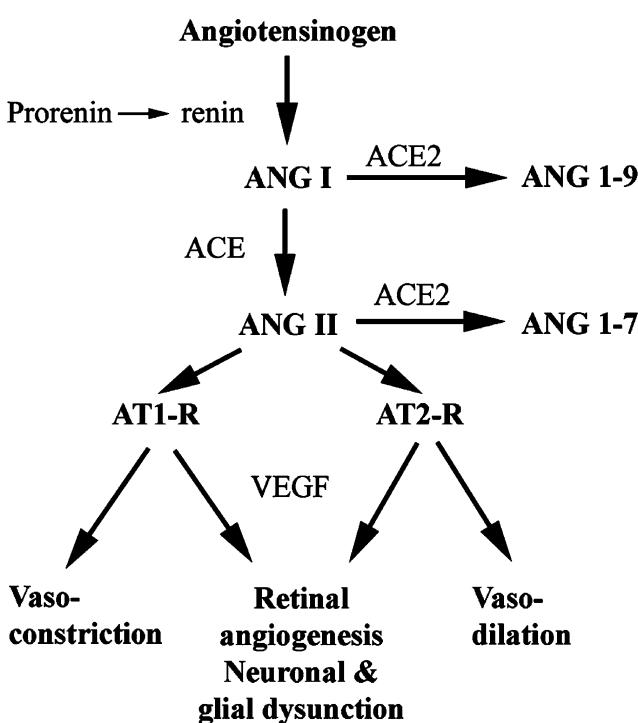


Fig. 2. Possible involvement of the renin–angiotensin system in vascular and neuronal and glial dysfunction in diabetic retinopathy. ANG, angiotensin; ACE, angiotensin-converting enzyme; AT1-R, angiotensin type 1 receptor; AT2-R, angiotensin type 2 receptor; VEGF, vascular endothelial growth factor.

3. The circulating RAS and diabetic retinopathy

A pathogenetic role for the RAS in diabetic retinopathy comes from early reports that plasma

prorenin is increased in patients with this condition (Franken et al., 1988; Luetscher, Kraemer, Wilson, Schwartz, & Bryer-Ash, 1985). In a study of 223 diabetic patients, elevations in plasma prorenin could be correlated with the severity of diabetic retinopathy (Franken et al., 1988). Subsequent studies have largely supported these findings (Allen et al., 1996; Deinum et al., 1999), and indicated that plasma prorenin might be unaltered during the progression from no retinopathy to background retinopathy, but become elevated from the onset of pre-PDR (Allen et al., 1996; Deinum et al., 1999). These studies have suggested extrarenal sources for plasma prorenin in diabetes, which may include the eye; however, it should be noted that the retina produces very small amounts of renin compared to other tissues (Berka et al., 1995). Overall, these findings have led to the suggestion that plasma prorenin might be a sensitive marker of renal microvascular disease in diabetes, as it rises before the onset of microalbuminuria (Allen et al., 1996). Elevated levels of prorenin have been reported in the vitreous fluids from patients with PDR compared to non-diabetic patients with spontaneous retinal detachment (Deinum, Derkx, Danser, & Schalekamp, 1990). Of interest is that a concentration gradient for prorenin and renin exists in ocular fluids, with prorenin highest in posterior vitreous followed by anterior vitreous and then aqueous humor, suggesting that the main source of ocular fluid prorenin is the posterior eye (Deinum et al., 1990).

4. The cellular location of the retinal renin–angiotensin system

The idea that a local retinal RAS exists in the eye and that it could contribute to pathology has been studied by a number of investigators. Although the kidney is the major site of renin and angiotensin production (Harris & Cheng, 1996), RASs also exist in tissues such as the adrenal gland, ovary, pituitary and thymus (Ganong, 1994; Rong, Wilkinson-Berka, & Skinner, 1999, 2001; Wilkinson-Berka, Kelly, Rong, Campbell, & Skinner, 2002). In the eye, RAS components are found in both ocular fluids and tissues (Danser et al., 1989; Deinum et al., 1990) in amounts that suggest local production. Ocular prorenin is present at levels 100 times higher than expected on the basis of the plasma protein content of ocular fluid (Danser et al., 1989). Similarly, in mice

and rats, retinal renin is present in levels that cannot be accounted for by plasma (Berka et al., 1995). However, retinal renin is in relatively low amounts compared with adrenal and kidney (Berka et al., 1995). ANG I and ANG II are found in the anterior uveal tract, neural retina, retinal pigmented epithelium (RPE) and choroid of the normal porcine eye at levels 5–100-fold higher, respectively, than can be accounted for by contamination from blood (Danser et al., 1994).

The cellular location of the RAS has been determined in the retina of both neonatal and adult rodents. Recent studies in the rat have demonstrated that all components of the RAS are evident as early as postnatal day 1, and persist throughout the first few weeks of postnatal retinal development and into the adult (Sarlos & Wilkinson-Berka, 2005). In the retina, RAS components are largely found in two sites: neurons and glia cells in the inner retina and blood vessels. Prorenin, renin and angiotensin are located in amacrine cells and ganglion cells, with renin also present in large macroglial Müller cells. In rats, mice and humans, renin is found along the full length of Müller cells, whereas prorenin is confined to Müller cell processes (Berka et al., 1995). Renin and ACE are also distributed throughout the retinal and choroidal vasculature. The finding of renin and angiotensin in glia and neurons suggests a role for these molecules not only in neuro-modulation but also in angiogenesis because these cell types are structurally aligned with the retinal vasculature (Tout, Chan-Ling, Hollander, & Stone, 1993). A local RAS is also found in human retina with studies utilizing real-time polymerase chain reaction (PCR) and RNase protection assays demonstrating that renin, angiotensinogen and ACE mRNA are synthesized in RPE/choroid and whole neural retina samples (Wagner et al., 1996).

The location of the angiotensin receptors in the retina is important when considering the possible functions of the retinal RAS. Autoradiographic binding techniques have demonstrated the presence of ANG II receptors in the retinal vasculature of various species (Ferrari-Dileo, Davis, & Anderson, 1991; Sato, Niwa, Himeno, Tsutumi, & Amemiya, 1993). More recently, AT1 and AT2 receptor transcripts have been identified in several ocular tissues including the retina (Brandt et al., 1994; Sarlos et al., 2003; Wheeler-Schilling, Kohler, Sautter, & Guenther, 1999). Interestingly, both AT1 and AT2 receptors are found on neurons and glial

cells and the vasculature (Nagai et al., 2005; Sarlos et al., 2003; Wheeler-Schilling et al., 1999). The finding that the AT2 receptor predominates in the developing retina (Sarlos et al., 2003) is consistent with the view that the AT2 receptor influences cell growth and differentiation in organ development (Csikos, Chung, & Unger, 1998; Timmermanns, Chiu, Herblin, Wong, & Smith, 1992).

Overall, the close anatomical arrangement of neurons, glia and blood vessels in the inner retina and the location of RAS components in these cell types suggest that ANG II may influence physiological processes within these cell types. It should be noted that it is also possible that components of the RAS are sequestered from the circulation into retinal cells to generate ANG II, which acts locally to modulate blood flow and cell growth.

5. Angiotensin and retinal vascular pathology

5.1. Pericytes

Retinal capillaries are comprised of a single layer of endothelial cells bounded by pericytes, and both cell types are covered by a common basement membrane (Hirschi & D'Amore, 1996). In diabetes, one of the earliest responses to hyperglycaemia is pericyte apoptosis (Beltramo, Berrone, Buttiglieri, & Porta, 2004). Pericytes are structurally and functionally aligned with vascular smooth muscle cells (VSMC), and play an important role in vessel patency by regulating vascular tone and providing structural support (Hirschi & D'Amore, 1996). Pericytes are viewed to be a regulator of angiogenesis as they are recruited to newly formed vessels and release anti-angiogenic agents (Hirschi & D'Amore, 1996). Hyperglycaemic-induced pericyte apoptosis is therefore a prerequisite for more severe vascular damage in the retina including vascular leakage, endothelial cell death, hypoxia and angiogenesis.

Angiotensin has distinct effects on VSMCs, inducing cell growth and proliferation and the deposition of extracellular matrix proteins (Kato et al., 1991; Tamura et al., 1998) via the stimulation of growth factors such as transforming growth factor (TGF) β 1 (Gibbons & Dzau, 1994), platelet-derived growth factor (PDGF) (Deguchi, Makuuchi, Nakaoka, Collins, & Takuwa,

1999), vascular endothelial growth factor (VEGF) (Williams, Baker, Gallacher, & Lodwick, 1995), insulin-like growth factor (Gustafsson, Andersson, Chen, Magnusson, & Arnqvist, 1999) and connective tissue growth factor (Ruperez, Lorenzo et al., 2003; Ruperez, Ruiz-Ortega et al., 2003). In general, these effects are mediated by the AT1 receptor. The actions of the AT2 receptor in pericyte/VSMC growth and migration are not fully understood. There is evidence that in the vascular wall of adults, the AT2 receptor might oppose the actions of the AT1 receptor, eliciting an anti-proliferative effect on aberrant VSMC growth by inducing pro-apoptotic pathways (Suzuki et al., 2002).

The effects of ANG II on retinal pericytes have been less extensively studied. There is evidence that ANG II induces pericyte hypertrophy (Schonfelder, Hofer, Paul, & Funk, 1998) and contraction (Ferrari-Dileo, Davis, & Anderson, 1996). Recently, angiotensin has been reported to activate voltage-dependent calcium channels in retinal microvascular cells via the AT1 receptor (Kawamura et al., 2004). Relevant to diabetic retinopathy is the finding that ANG II causes functional uncoupling of pericytes from retinal microvessels (Kawamura et al., 2004). ANG II has been shown to induce migration of retinal pericytes, which is inhibited by selective AT1 receptor blockade (Nadal, Scicli, Carbini, Nussbaum, & Scicli, 1999). In the same study, blockade of the AT2 receptor stimulated migration, indicating that the AT2 receptor might oppose the pro-migratory effects of the AT1 receptor. ANG II's pro-migratory effects on retinal pericytes might involve PDGF and TGF- β . ANG II has been reported to up-regulate PDGF gene expression in bovine retinal pericytes, which can be attenuated with the AT1 receptor blocker telmisartan (Amano, Yamagishi, Inagaki, & Okamoto, 2003). ANG II stimulation of pericyte migration can be partially attenuated by neutralising antibodies to TGF- β 1/2/3 and abolished by PDGF-BB at low concentrations (Nadal, Scicli, Carbini, & Scicli, 2002). However, other studies of cultured retinal pericyte have reported that ANG II does not stimulate the release of TGF- β (Limone et al., 2002). ANG II has also been reported to stimulate the generation of oxygen free radicals (Yamagishi et al., 2003) and VEGF expression in retinal pericytes, which is mediated by the AT1 receptor (Otani et al., 2000; Yamagishi et al., 2003).

5.2. Angiogenesis

ANG II's pro-angiogenic abilities were first demonstrated in the rabbit cornea (Fernandez, Twickler, & Mead, 1985) and chick chorioallantoic membrane (Le Noble, Hekking, Van Straaten, Slaaf, & Struyker Boudier, 1991). Subsequent studies have reported that ANG II promotes pathological angiogenesis in tumours (Egami et al., 2003; Volpert et al., 1996) and coronary capillaries (Jesmin, Hattori, Sakuma, Mowa, & Kitabatake, 2002). In terms of retinal angiogenesis, the oxygen-induced retinopathy (OIR) model is frequently used to evaluate the efficacy of anti-angiogenic strategies (Chavakis et al., 2002; Penn, Tolman, & Lowery, 1993; Sarlos et al., 2003; Smith et al., 1994). Pathological angiogenesis in OIR occurs when neonatal rodents are exposed to a period of hyperoxia followed by hypoxia (Sarlos et al., 2003; Smith et al., 1994). In rats with OIR, the ocular RAS becomes up-regulated, with elevated levels of renin (Moravski et al., 2000) and AT1 and AT2 receptor expression (Sarlos et al., 2003). ACE inhibitors and AT1 receptor blockers administered during the period of retinal hypoxia attenuate preretinal pathological angiogenesis to almost sham levels (Lonchampt, Pennel, & Duhault, 2001; Moravski et al., 2000; Nagai et al., 2005) and reduce inflammation (Nagai et al., 2005). In addition, ACE inhibitors reduce OIR in mice when administered during the hyperoxic period and this is associated with down-regulation of endothelin 1 (Tadesse, Yan, Yossuck, & Higgins, 2001).

There are conflicting reports regarding the role of the AT2 receptor in OIR-induced angiogenesis. The AT2 receptor blocker, PD123319, has been reported to have no effect on OIR in mice (Lonchampt et al., 2001) and attenuate angiogenesis in rats with OIR (Sarlos et al., 2003). The reasons for the anti-angiogenic effects of PD123319 in rats may be due to its continuous infusion by miniosmotic pump (Sarlos et al., 2003), which is known to be necessary to effectively block the AT2 receptor (Cao, Dean, Wu, Casley, & Cooper, 1999). It is also possible that differences in the abundance of AT1 and AT2 receptors in the mouse and rat retina might account for the discrepancies between the studies.

RAS blockade has been examined in the developing retina. In rodents, the retinal vasculature develops postnatally and is complete by approximately 2 weeks of life (Connolly, Hores, Smith, & D'Amore, 1988).

Although AT1 and AT2 receptor inhibition reduces pathological retinal angiogenesis in neonatal rodents with OIR, it has no effect on developmental retinal angiogenesis in neonatal sham animals when administered between postnatal days 7 and 12 (mice) or 12 and 18 (rats) (Lonchampt et al., 2001; Moravski et al., 2000; Nagai et al., 2005). However, recent studies indicate that the AT2 receptor may be more important in developmental retinal angiogenesis than the AT1 receptor. In the rat retina, AT2 receptor binding is generally higher than the AT1 receptor during early postnatal development at postnatal days 1–7 (Sarlos et al., 2003), a finding that is in agreement with other studies where the AT2 receptor is viewed to influence organ development (Grady, Sechi, Griffin, Schambelan, & Kalinyak, 1991). In 7-day-old rats, the retinal vasculature is in the early phases of development. In these animals, ACE inhibition but not AT1 receptor antagonism reduced vascular density in the central, mid and peripheral retina. As blockade of ACE may influence both the AT1 and AT2 receptors, it is possible that the anti-angiogenic effects of ACE inhibition in the neonatal rat are related to down-regulation of the AT2 receptor.

The pro-angiogenic actions of ANG II in the retina of OIR animals are linked to VEGF, a potent vascular permeability, angiogenic and survival factor for endothelial cells (reviewed in Ferrara, 1999). In the normal retina, VEGF influences the development of the retinal microvasculature (Provis et al., 1997; Stone et al., 1995) and photoreceptors (Yourey, Gohari, Su, & Alderson, 2000), and might confer neuroprotection (Bocker-Meffert et al., 2002; Sandercoe, Geller, Hendrickson, Stone, & Provis, 2003). Components of the VEGF family are present in vascular and neuroglial components of the retina. VEGF receptor-1 (VEGFR-1) has been identified in retinal pericytes (Witmer et al., 2002), and VEGF and VEGF receptor-2 (VEGFR-2) protein and mRNA in blood vessels, astrocytes, Müller cells, ganglion cells and the RPE (Gilbert et al., 1998; Moravski et al., 2000; Stone et al., 1995). In diabetes, VEGF is implicated in breakdown of the blood–retinal barrier, which occurs early and can persist during the course of the disease (Aiello et al., 1994; Funatsu et al., 2003; Gilbert et al., 2000; Murata et al., 1995).

In general, pharmacological blockade of the RAS either at the level of ACE or the angiotensin

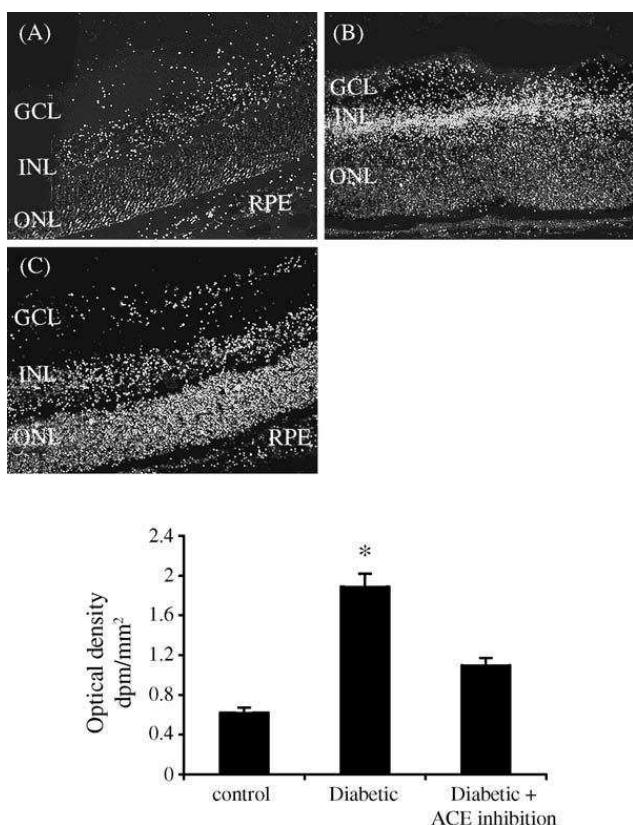


Fig. 3. Three micrometre paraffin section of retina from non-diabetic and diabetic rats showing vascular endothelial growth factor (VEGF) expression. Graph represents quantitation of VEGF expression in the inner retina (GCL, inner plexiform layer and INL). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. (A) Untreated non-diabetic; (B) untreated diabetic; (C) diabetic + angiotensin-converting enzyme (ACE) inhibition. In rat retina, VEGF expression is observed in the INL, RPE and to a lesser extent in the GCL. VEGF expression is increased with diabetes (B) and reduced in diabetic rats treated with an ACE inhibitor. Magnification $\times 150$. Values are mean \pm S.E.M. $N=6$ –8 rats per group. * $P<0.0001$ compared to all groups. Images were captured using Analytical Imaging Software (Ont., Canada) and the optical density value of each pixel of digitized image converted into dpm/mm² (disintegration per minute of 125 I per mm² of tissue) (Moravski et al., 2000, 2003).

receptors reduces pathological retinal angiogenesis, and is accompanied by down-regulation of VEGF and VEGFR-2. In vivo, ACE inhibition reduces the rise in retinal VEGF and VEGFR-2 that occurs in Sprague–Dawley rats (Gilbert et al., 2000) and transgenic (mRen-2)27 rats (Moravski et al., 2003) with streptozotocin diabetes (Fig. 3), and rats with OIR (Moravski et al., 2000). In these situations, ACE inhibition also attenuates vascular pathology including

vascular leakage, the proliferation of endothelial cells (Moravski et al., 2003) and angiogenesis (Moravski et al., 2000). Interestingly, although blockade of the AT1 receptor attenuates retinal angiogenesis in rats with OIR, it does not reduce the rise in retinal VEGF and VEGFR-2 mRNA in the inner retina (Moravski et al., 2000). The reasons for this finding are not completely understood. There is evidence that the AT2 receptor also influences pathological angiogenesis in OIR. Blockade of the AT2 receptor in rats with OIR was shown to reduce retinal angiogenesis and expression of VEGF, VEGFR-2 and angiopoietin 2 (Sarlos et al., 2003). In terms of diabetes, both AT1 and AT2 receptor blockade in Sprague–Dawley rats attenuates the rise in retinal VEGF expression (Zhang, Lassila, Cooper, & Cao, 2004). Similarly, infusion of ANG II into rats elevates retinal VEGF expression, which can be reduced with either AT1 or AT2 receptor blockade (Zhang et al., 2004). Overall, these findings suggest that in the retina, both the AT1 and AT2 receptor can modulate VEGF expression.

6. Angiotensin 1–7 and ACE2

Emerging evidence suggests that the ANG 1–7 and ACE2 arm of the RAS cascade may oppose the classical actions of ANG II and ACE (Carey & Siragy, 2003) (Fig. 2). ANG 1–7 can be formed directly from ANG I by the actions of several peptidases such as neutral endopeptidase, prolylendopeptidase or carboxypeptidase. Recent evidence indicates that ACE2 may be a major pathway for the formation of ANG 1–7 (Fig. 2). In terms of haemodynamics, ANG 1–7 may oppose the vasoconstrictor actions of ANG II by the stimulation of nitric oxide and vasodilator prostaglandins (Jaiswal, Diz, Chappell, Khosla, & Ferrario, 1992; Li, Chappell, Ferrario, & Brosnihan, 1997). There is also evidence that ANG 1–7 can potentiate the vasodilator actions of bradykinin and its B2 receptor (Brosnihan, Li, & Ferrario, 1996; Paula, Lima, Khosla, & Santos, 1995). Apart from its vasodepressor actions, ANG 1–7 may also have anti-proliferative effects in vascular cells. Whether ANG 1–7 is anti-angiogenic or alters vascular patency in the normal and diabetic retina is unknown; however, it is interesting to note that ACE2 is located in the inner nuclear layer and photoreceptors cells of the rat retina (Tikellis et al., 2004). In this same

study, ACE2 expression was reduced in the diabetic retina while ACE expression was increased (Tikellis et al., 2004). Whether the retinal ANG1-7/ACE2 pathway counter-regulates the vasoconstrictor and growth-promoting properties of ANG II in the diabetic retina requires further investigation.

7. The streptozotocin diabetic transgenic (mRen-2)27 rat

Experimental models of diabetes have been used to evaluate the relationship between the RAS and retinopathy. In diabetic Sprague–Dawley rats, ACE inhibition reduced glucose accumulation in retinal tissue (Zhang et al., 2004), ameliorated retinal hyperpermeability (Gilbert et al., 2000) and restored retinal blood flow (Horio et al., 2004). Evaluation of the effect of RAS blockade on more severe diabetic retinal pathology has been hampered by the absence of a diabetic animal model that exhibits pre-PDR or PDR (Engerman et al., 1982); most diabetic rodent models develop only early retinal changes, such as pericyte loss, capillary dilation and increased basement membrane thickening, even after a year of diabetes (Su et al., 2000). The transgenic (mRen-2)27 rat has enabled studies of RAS in advanced diabetic retinopathy and nephropathy (Berka et al., 1995; Kelly, Wilkinson-Berka, Allen, Cooper, & Skinner, 1998; Rong et al., 1999, 2001; Wilkinson-Berka, Kelly, Koerner et al., 2002).

The enzyme renin is encoded by two genes, *Ren-1* and *Ren-2*, which have differential expression patterns in various species. The transgenic (mRen-2)27 rat was developed by the introduction of the murine *Ren-2* gene into the genome of a Sprague–Dawley rat (Mullins, Peters, & Ganten, 1990). The transgenic (mRen-2)27 rat expresses the *Ren-2* gene in addition to the endogenous rat *Ren-1* gene. This animal exhibits fulminant hypertension and overexpresses renin and angiotensin in tissues such as thymus, gut, adrenal, ovary and eye (Berka et al., 1995; Kelly et al., 1998; Rong et al., 1999, 2001; Wilkinson-Berka, Kelly, Rong et al., 2002). When made diabetic with streptozotocin, the transgenic (mRen-2)27 rat progressively develops a severe nephropathy, with similarities to severe human diabetic nephropathy (Kelly et al., 2000, 2004, 1998), including a decline in glomerular filtration rate, severe

glomerulosclerosis, tubular apoptosis and occasional medullary necrosis. The kidney lesion is associated with up-regulation of renin gene expression in juxtaglomerular cells and proximal tubules and the renal pathology can be attenuated by blockade of the RAS, suggesting a pathogenetic role for the site-specific RAS in the setting of diabetes. Hypertension and nephropathy are known risk factors for the development of diabetic retinopathy (Knowler, Bennett, & Ballantine, 1980). Examination of ocular pathology in the transgenic (mRen-2)27 rat revealed alterations to the retinal and iris vasculature after the appearance of the severe kidney lesion (Moravski et al., 2003). Ocular renin is increased in the diabetic (mRen-2)27 rat and the number of proliferating endothelial cells is elevated in both the retina and iris. Similar to diabetic nephropathy, the ocular lesion can be attenuated with blockade of the RAS. Overall, these findings implicate the ocular RAS in diabetic retinal pathology, and suggest that blockade of the RAS confers retinoprotection.

Components of the RAS are found in neurons and glia; however, the functions of the RAS at these sites are not fully understood. The RAS might influence electrophysiological function in the normal retina. RAS blockade has been reported to modulate the b-wave and a-wave of the electroretinogram (Jacobi, Osswald, Jurklies, & Zrenner, 1994; Jurklies, Kohler, Eikermann, & Zrenner, 1994), an effect that can be reversed by administration of ANG II. There are few reports examining these effects in diabetes, but a recent study in streptozotocin diabetic Sprague–Dawley rats indicates that ACE inhibition attenuates the diabetes-induced losses in photoreceptor-P3, postreceptor-P2 and oscillatory potential amplitudes of the electroretinogram (Bui, Armitage, Tolcos, Cooper, & Vingrys, 2003). Similarly, in diabetic spontaneously hypertensive rats (SHRs), AT1 receptor blockade attenuates the latencies of oscillatory potentials, an effect that is independent of blood pressure (Nagisa, Shintani, & Nakagawa, 2001).

8. Hypertension

Hypertension is a known risk factor for the development of microvascular disease in diabetic retinopathy (Knowler et al., 1980; Wong et al., 2002). For instance, hypertension in spontaneously diabetic obese rhesus

monkeys is associated with intraretinal haemorrhages, areas of tissue non-perfusion and also a reduction in photoreceptors and retinal function as measured by the electroretinogram (Johnson et al., 2005). There is evidence that hypertension or its in vitro counterpart, mechanical stretch, increases the expression of the RAS and VEGF system (Malhotra, Sadoshima, Brosius, & Izumo, 1999). In the eye, mechanical stretch up-regulated VEGF expression in cultured RPE (Seko, Fujikura, Pang, Tokoro, & Shimokawa, 1999) and in bovine retinal endothelial cells (Suzuma et al., 2001). In addition, VEGFR-2 mRNA was found to be increased in the retinae of SHRs compared to normotensive rats (Suzuma et al., 2001). It is therefore possible that the reduction in VEGF expression reported in ROP (Lonchampt et al., 2001; Moravski et al., 2000) and diabetic retinopathy (Moravski et al., 2003) following RAS blockade, may to some extent be due to the anti-hypertensive effects of this therapy rather than inhibition of the growth factor effects of ANG II. It should be noted that streptozotocin diabetic SHRs do not develop endothelial cell proliferation in the retina and iris (Moravski et al., 2003) or severe nephropathy (Kelly et al., 1998) when made diabetic with streptozotocin, whereas, in direct contrast, lesions develop in the eyes and kidneys of diabetic transgenic (mRen-2)27 rats with comparable levels of blood pressure to the SHR; thus, hypertension might contribute to but not be a major factor in the development of diabetic retinopathy (Moravski et al., 2003).

9. Clinical studies

Studies in patients with type 2 diabetes suggest that ACE inhibition and AT1 receptor blockade have little or no effect on retinal vascular abnormalities. For instance, the UK Prospective Diabetes Study Group reported that the ACE inhibitor captopril and the β -blocker atenolol were equally effective in reducing blood pressure; however, there was no difference in the progression of retinopathy between the two treatments (UKPDS Study Group, 1998). The Appropriate Blood Pressure Control in Diabetes (ABCD) trial reported that over a 5-year follow-up period there was no difference in the progression of retinopathy between patients treated with either nisoldipine or the ACE inhibitor enalapril (Estacio, Jeffers, Gifford, & Schrier,

2000). The Heart Outcomes Prevention Evaluation (HOPE)/MICRO-HOPE trial evaluated retinopathy on the basis for the need for laser photocoagulation (HOPE Study, 2000) and found that the ACE inhibitor ramipril had a non-significant relative risk reduction in the requirement for laser photocoagulation (HOPE Study, 2000). In small studies of type 2 diabetic patients, ACE inhibition did not exert a beneficial effect on the progression of retinopathy (Pradhan et al., 2002), and AT1 receptor blockade with losartan did not improve macular oedema and the number of hard exudates in patients with diabetic maculopathy (Knudsen et al., 2003). However, in a study of 19 normotensive type 2 diabetic patients with diabetic macular oedema who received oral lisinopril therapy for 2 months, the ACE inhibitor lisinopril therapy reduced macular thickness (Funatsu et al., 2003).

Studies evaluating RAS blockade in type 1 diabetes have mainly reported an improvement in diabetic retinopathy. Small studies of normotensive type 1 diabetic patients have shown that ACE inhibition, arrested or delayed fluorescein retinal leakage (Larsen, Hommel, Parving, & Lund-Andersen, 1990), and improved the distribution of changes in retinal grades over a 2-year period (Chase et al., 1993). In contrast, in hypertensive patients with type 1 diabetes and background retinopathy, captopril did not attenuate breakdown of the blood-retinal barrier (Engler, Parving, Mathiesen, Larsen, & Lund-Andersen, 1991). In the EURODIAB Controlled Trial of Lisinopril in Insulin-dependent Diabetes Mellitus (EUCLID), Chaturvedi, Sjolie, Stephenson, Abrahamian, and Keipes (1998) reported that in patients that were not hypertensive and either normoalbuminuric (85% of patients) or microalbuminuric, lisinopril decreased the progression of retinopathy by two or more grades, and the progression to proliferative retinopathy. It was found that diabetic patients with better glycaemic control had the most benefit from ACE inhibition (Chaturvedi et al., 1998). Confirmation of the retinoprotective benefits of the EUCLID trial will await larger studies such as the Diabetic Retinopathy Candesartan Trial (DIRECT), a 4-year multicentre trial evaluating the effects of the AT1 receptor blockade on retinopathy in both type 1 and type 2 diabetic patients (Sjolie & Chaturvedi, 2002). Of interest is whether there will be any differential effects of RAS blockade on patients with type 1 and type 2 diabetes.

10. Summary

ANG II is a pathogenic factor in diabetes, contributing to angiogenesis, vascular leakage, fibrosis, inflammation and the up-regulation of growth factors. To date, findings from in vitro studies and experimental models of ischemic retinopathy indicate that ANG II contributes to retinal pathology. In terms of diabetic retinopathy, there is evidence that ANG II promotes vascular, neuronal and glial disease, and that these events can be improved by RAS blockade. Further studies are required to determine if RAS blockade can confer retinoprotection and arrest the progression of diabetic retinopathy in patients with both type 1 and type 2 diabetes.

Acknowledgements

The author thanks the National Health and Medical Research Council of Australia and Juvenile Diabetes Research Foundation.

References

- Aguilera, G., & Kiss, A. (1996). Regulation of the hypothalamic–pituitary–adrenal axis and vasopressin secretion. Role of angiotensin II. *Adv. Exp. Med. Biol.*, 396, 105–112.
- Aiello, L. M. (2003). Perspectives on diabetic retinopathy. *Am. J. Ophthalmol.*, 136, 122–135.
- Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., et al. (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N. Engl. J. Med.*, 331, 1480–1487.
- Allen, T. J., Cooper, M. E., Gilbert, R. E., Winikoff, J., Skinnier, S. L., & Jerums, G. (1996). Serum total renin is increased before microalbuminuria in diabetes. *Kidney Int.*, 50, 902–907.
- Allen, A. M., Yamada, H., & Mendelsohn, F. A. (1990). In vitro autoradiographic localization of binding to angiotensin receptors in the rat heart. *Int. J. Cardiol.*, 28, 25–33.
- Amano, S., Yamagishi, S., Inagaki, Y., & Okamoto, T. (2003). Angiotensin II stimulates platelet-derived growth factor-B gene expression in cultured retinal pericytes through intracellular reactive oxygen species generation. *Int. J. Tissue React.*, 25, 51–55.
- Beltramo, E., Berrone, E., Buttiglieri, S., & Porta, M. (2004). Thiamine and benfotiamine prevent increased apoptosis in endothelial cells and pericytes cultured in high glucose. *Diab. Metab. Res. Rev.*, 20, 330–336.
- Berka, J. L., Stubbs, A. J., Wang, D. Z.-M., Di Nicolantonio, R., Alcorn, D., Campbell, D. J., et al. (1995). Renin-containing Muller cells of the retina display endocrine features. *Invest. Ophthalmol. Vis. Sci.*, 36, 1450–1458.
- Bocker-Meffert, S., Rosenstiel, P., Rohl, C., Warneke, N., Held-Feindt, J., Sievers, J., et al. (2002). Erythropoietin and VEGF promote neural outgrowth from retinal explants in postnatal rats. *Invest. Ophthalmol. Vis. Sci.*, 43, 2021–2026.
- Brandt, C. R., Pumfrey, A. M., Micales, B., Bindley, C. D., Lyons, G. E., Sramek, S. J., et al. (1994). Renin mRNA is synthesized locally in rat ocular tissues. *Curr. Eye Res.*, 13, 755–763.
- Brosnihan, K. B., Li, P., & Ferrario, C. M. (1996). Angiotensin-(1–7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension*, 27, 523–528.
- Bui, B. V., Armitage, J. A., Tolcos, M., Cooper, M. E., & Vingrys, A. J. (2003). ACE inhibition salvages the visual loss caused by diabetes. *Diabetologia*, 46, 401–408.
- Burrell, L. M., Johnston, C. I., Tikellis, C., & Cooper, M. E. (2004). ACE2, a new regulator of the renin–angiotensin system. *Trends Endocrinol. Metab.*, 15, 166–169.
- Cao, Z., Dean, R., Wu, L., Casley, D., & Cooper, M. E. (1999). Role of angiotensin receptor subtypes in mesenteric vascular proliferation and hypertrophy. *Hypertension*, 34, 408–414.
- Cao, Z., Kelly, D. J., Cox, A., Casley, D., Forbes, J. M., Martinello, P., et al. (2000). Angiotensin type 2 receptor is expressed in the adult rat kidney and promotes cellular proliferation and apoptosis. *Kidney Int.*, 58, 2437–2451.
- Carey, R. M., & Siragy, H. M. (2003). Newly recognized components of the renin–angiotensin system: Potential roles in cardiovascular and renal regulation. *Endocr. Rev.*, 24, 261–271.
- Chase, H. P., Garg, S. K., Harris, S., Hoops, S., Jackson, W. E., & Holmes, D. L. (1993). Angiotensin-converting enzyme inhibitor treatment for young normotensive diabetic subjects: A two-year trial. *Ann. Ophthalmol.*, 25, 284–289.
- Chaturvedi, M., Sjolie, A. K., Stephenson, J. M., Abrahamian, H., & Keipes, M. (1998). Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. *Lancet*, 351, 28–31.
- Chavakis, E., Riecke, B., Lin, J., Linn, T., Bretzel, R. G., Preissner, K. T., et al. (2002). Kinetics of integrin expression in the mouse model of proliferative retinopathy and success of secondary intervention with cyclic RGD peptides. *Diabetologia*, 45, 262–267.
- Chung, O., Kuhl, H., Stoll, M., & Unger, T. (1998). Physiological and pharmacological implications of AT₁ versus AT₂ receptors. *Kidney Int.*, 54, S95–S99.
- Connolly, S. E., Hores, T. A., Smith, L. E., & D’Amore, P. A. (1988). Characterization of vascular development in the mouse retina. *Microvasc. Res.*, 36, 275–290.
- Csikos, T., Chung, O., & Unger, T. (1998). Receptors and their classification: Focus on angiotensin II and the AT₂ receptor. *J. Hum. Hypertens.*, 12, 311–318.
- Culman, J., Hohle, S., Qadri, F., Edling, O., Blume, A., Lebrun, C., et al. (1995). Angiotensin as neuromodulator/neurotransmitter in central control of body fluid and electrolyte homeostasis. *Clin. Exp. Hypertens.*, 17, 281–293.
- Danser, A. H., Derkx, F. H., Admiraal, P. J., Deinum, J., de Jong, P. T., & Schalekamp, M. A. (1994). Angiotensin levels in the eye. *Invest. Ophthalmol. Vis. Sci.*, 35, 1008–1018.

- Danser, A. H. J., van den Dorpel, M. A., Deinum, J., Derkx, F. H. M., Franken, A. A. M., Peperkamp, E., et al. (1989). Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J. Clin. Endocrinol. Metab.*, 68, 160–167.
- Deguchi, J., Makuuchi, M., Nakaoka, T., Collins, T., & Takuwa, Y. (1999). Angiotensin II stimulates platelet-derived growth factor-B chain expression in newborn rat vascular smooth muscle cells and neointimal cells through Ras, extracellular signal-regulated protein kinase, and c-Jun N-terminal protein kinase mechanisms. *Circ. Res.*, 85, 565–574.
- Deinum, J., Derkx, F. H., Danser, A. H., & Schalekamp, M. A. (1990). Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology*, 126, 1673–1682.
- Deinum, J., Tarnow, L., van Gool, J. M., de Bruin, R. A., Derkx, F. H. M., Schalekamp, M. A. D. H., et al. (1999). Plasma renin and prorenin and renin gene variation in patients with insulin-dependent diabetes mellitus and nephropathy. *Nephrol. Dial. Transplant*, 14, 1904–1911.
- Egami, K., Murohara, T., Shimada, T., Sasaki, K.-i., Shintani, S., Sugaya, T., et al. (2003). Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. *J. Clin. Invest.*, 112, 67–75.
- Engerman, R., Finkelstein, D., Aguirre, G., Diddie, K. R., Fox, R. R., Frank, R. N., et al. (1982). Ocular complications. *Diabetes*, 31, 82–88.
- Engler, C. B., Parving, H. H., Mathiesen, E. R., Larsen, M., & Lund-Andersen, H. (1991). Blood-retina barrier permeability in diabetes during acute ACE-inhibition. *Acta Ophthalmol. (Copenh.)*, 69, 581–585.
- Estacio, R. O., Jeffers, B. W., Gifford, N., & Schrier, R. W. (2000). Effect of blood pressure control on diabetic microvascular complications in patients with hypertension and type 2 diabetes. *Diab. Care*, 23(Suppl. 2), B54–B64.
- Fabiani, M. E., Hawkes, D. J., Frauman, A. G., Tikellis, C., Johnston, C. I., & Wilkinson-Berka, J. L. (2003). Regulation of angiotensin II receptors in the prostate of the transgenic (mRen-2)27 rat: Effect of angiotensin-converting enzyme inhibition. *Int. J. Biochem. Cell Biol.*, 35, 973–983.
- Fernandez, L. A., Twickler, J., & Mead, A. (1985). Neovascularization produced by angiotensin II. *J. Lab. Clin. Med.*, 105, 141–145.
- Ferrara, N. (1999). Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int.*, 56, 794–814.
- Ferrari-Dileo, G., Davis, E. B., & Anderson, D. R. (1991). Angiotensin II binding receptors in retinal and optic nerve head blood vessels. An autoradiographic approach. *Invest. Ophthalmol. Vis. Sci.*, 32, 21–26.
- Ferrari-Dileo, G., Davis, E. B., & Anderson, D. R. (1996). Glaucoma, capillaries and pericytes. 3. Peptide hormone binding and influence on pericytes. *Ophthalmologica*, 210, 269–275.
- Frank, R. N. (2004). Diabetic retinopathy. *N. Engl. J. Med.*, 350, 48–58.
- Franken, A. A., Derkx, F. H., Schalekamp, M. A., Man in't Veld, A. J., Hop, W. C., van Rens, E. H., et al. (1988). Association of high plasma prorenin with diabetic retinopathy. *J. Hypertens. Suppl.*, 6, S461–S463.
- Funatsu, H., Yamashita, H., Ikeda, T., Mimura, T., Eguchi, S., & Hori, S. (2003). Vitreous levels of interleukin-6 and vascular endothelial growth factor are related to diabetic macular edema. *Ophthalmology*, 110, 1690–1696.
- Ganong, W. F. (1994). Origin of the angiotensin II secreted by cells. *Proc. Soc. Exp. Biol. Med.*, 205, 213–219.
- Gibbons, G. H., & Dzau, V. J. (1994). The emerging concept of vascular remodeling. *N. Engl. J. Med.*, 330, 1431–1438.
- Gilbert, R. E., Kelly, D. J., Cox, A. J., Wilkinson-Berka, J. L., Rumble, J. R., Osicka, T., et al. (2000). Angiotensin converting enzyme inhibition reduces retinal overexpression of vascular endothelial growth factor and hyperpermeability in experimental diabetes. *Diabetologia*, 43, 1360–1367.
- Gilbert, R. E., Krum, H., Wilkinson-Berka, J., & Kelly, D. J. (2003). The renin-angiotensin system and the long-term complications of diabetes: Pathophysiological and therapeutic considerations. *Diab. Med.*, 20, 607–621.
- Gilbert, R. E., Vranes, D., Berka, J. L., Kelly, D. J., Cox, A., Wu, L. L., et al. (1998). Vascular endothelial growth factor and its receptors in control and diabetic rat eyes. *Lab. Invest.*, 78, 1017–1027.
- Grady, E. F., Sechi, L. A., Griffin, C. A., Schambelan, M., & Kalinyak, J. E. (1991). Expression of AT2 receptors in the developing rat fetus. *J. Clin. Invest.*, 88, 921–933.
- Gustafsson, T., Andersson, P., Chen, Y., Magnusson, J. O., & Arqvist, H. J. (1999). Interaction of angiotensin II and the insulin-like growth factor system in vascular smooth muscle cells. *Am. J. Physiol.*, 277, H499–H507.
- Harris, R. C., & Cheng, H. F. (1996). The intrarenal renin-angiotensin system: A paracrine system for the local control of renal function separate from the systemic axis. *Exp. Nephrol.*, 4(Suppl. 1), 2–7.
- Hirschi, K. K., & D'Amore, P. A. (1996). Pericytes in the microvasculature. *Cardiovasc. Res.*, 32, 687–698.
- HOPE Study. (2000). Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: Results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators. *Lancet*, 355, 253–259.
- Horio, N., Clermont, A. C., Abiko, A., Abiko, T., Shoelson, B. D., Bursell, S. E., et al. (2004). Angiotensin AT(1) receptor antagonism normalizes retinal blood flow and acetylcholine-induced vasodilation in normotensive diabetic rats. *Diabetologia*, 47, 113–123.
- Ito, M., Oliverio, M. I., Mannon, P. J., Best, C. F., Maeda, N., Smithies, O., et al. (1995). Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc. Natl. Acad. Sci. U.S.A.*, 92, 3521–3525.
- Jacobi, P. C., Osswald, H., Jurkliess, B., & Zrenner, E. (1994). Neuromodulatory effects of the renin-angiotensin system on the cat electroretinogram. *Invest. Ophthalmol. Vis. Sci.*, 35, 973–980.
- Jaiswal, N., Diz, D. I., Chappell, M. C., Khosla, M. C., & Ferrario, C. M. (1992). Stimulation of endothelial cell prostaglandin production by angiotensin peptides. Characterization of receptors. *Hypertension*, 19, II49–II55.
- Jesmin, S., Hattori, Y., Sakuma, I., Mowa, C. N., & Kitabatake, A. (2002). Role of ANG II in coronary capillary angiogenesis at the insulin-resistant stage of a NIDDM rat model. *Am. J. Physiol. Heart Circ. Physiol.*, 283, H1387–H1397.

- Johnson, M. A., Lutty, G. A., McLeod, D. S., Otsuji, T., Flower, R. W., Sandagar, G., et al. (2005). Ocular structure and function in an aged monkey with spontaneous diabetes mellitus. *Exp. Eye Res.*, 80, 37–42.
- Joussen, A. M., Poulaki, V., Le, M. L., Koizumi, K., Esser, C., Janicki, H., et al. (2004). A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB J.*, 18, 1450–1452.
- Jurlikes, B., Kohler, K., Eikermann, J., & Zrenner, E. (1994). Angiotensin II-like immunoreactivity in the retina of some mammalian species. *Ger. J. Ophthalmol.*, 3, 37–42.
- Kato, H., Suzuki, H., Tajima, S., Ogata, Y., Tominaga, T., Sato, A., et al. (1991). Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J. Hypertens.*, 9, 17–22.
- Kawamura, H., Kobayashi, M., Li, Q., Yamanishi, S., Katsumura, K., Minami, M., et al. (2004). Effects of angiotensin II on the pericyte-containing microvasculature of the rat retina. *J. Physiol.*, 561, 671–683.
- Kelly, D. J., Skinner, S. L., Gilbert, R. E., Cox, A. J., Cooper, M. E., & Wilkinson-Berka, J. L. (2000). Effects of endothelin or angiotensin II receptor blockade on diabetes in the transgenic (mRen-2)27 rat. *Kidney Int.*, 57, 1882–1894.
- Kelly, D. J., Stein-Oakley, A., Zhang, Y., Wassef, L., Maguire, J., Koji, T., et al. (2004). Fas-induced apoptosis is a feature of progressive diabetic nephropathy in transgenic (mRen-2)27 rats: Attenuation with renin–angiotensin blockade. *Nephrol. (Carlton)*, 9, 7–13.
- Kelly, D. J., Wilkinson-Berka, J. L., Allen, T. A., Cooper, M. E., & Skinner, S. L. (1998). A new model of diabetic nephropathy with progressive renal impairment in the transgenic (mRen-2)27 rat. *Kidney Int.*, 54, 343–352.
- Kim, S., & Iwao, H. (2000). Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol. Rev.*, 52, 11–34.
- Klein, R., Klein, B., & Moss, S. (1992). Epidemiology of proliferative diabetic retinopathy. *Diab. Care*, 15, 1875–1891.
- Klein, R., Klein, B. E., Moss, S. E., & Cruickshanks, K. J. (1998). The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes. *Ophthalmology*, 105, 1801–1815.
- Klein, R., Klein, B. E., Moss, S. E., Wong, T. Y., Hubbard, L., Cruickshanks, K. J., et al. (2004). The relation of retinal vessel caliber to the incidence and progression of diabetic retinopathy: XIX: The Wisconsin Epidemiologic Study of Diabetic Retinopathy. *Arch. Ophthalmol.*, 122, 76–83.
- Knowler, W. C., Bennett, P. H., & Ballantine, E. J. (1980). Increased incidence of retinopathy in diabetics with elevated blood pressure. A six-year follow-up study in Pima Indians. *N. Engl. J. Med.*, 302, 645–650.
- Knudsen, S. T., Bek, T., Poulsen, P. L., Hove, M. N., Rehling, M., & Mogensen, C. E. (2003). Effects of losartan on diabetic maculopathy in type 2 diabetic patients: A randomized, double-masked study. *J. Intern. Med.*, 254, 147–158.
- Larsen, M., Hommel, E., Parving, H. H., & Lund-Andersen, H. (1990). Protective effect of captopril on the blood–retina barrier in normotensive insulin-dependent diabetic patients with nephropathy and background retinopathy. *Graefes Arch. Clin. Exp. Ophthalmol.*, 228, 505–509.
- Le Noble, F. A., Hekking, J. W., Van Straaten, H. W., Slaaf, D. W., & Stryker Boudier, H. A. (1991). Angiotensin II stimulates angiogenesis in the chorio-allantoic membrane of the chick embryo. *Eur. J. Pharmacol.*, 195, 305–306.
- Li, P., Chappell, M. C., Ferrario, C. M., & Brosnihan, K. B. (1997). Angiotensin-(1–7) augments bradykinin-induced vasodilation by competing with ACE and releasing nitric oxide. *Hypertension*, 29, 394–400.
- Limone, P., Berardi, C., Pomero, F., Del Rizzo, P., Allione, A., Beltramo, E., et al. (2002). Failure of angiotensin II and insulin to stimulate transforming growth factor-beta1. Release from cultured bovine retinal pericytes. *Diab. Metab.*, 28, 499–503.
- Lonchampt, M., Pennel, L., & Duhault, J. (2001). Hyperoxia/normoxia-driven retinal angiogenesis in mice: A role for angiotensin II. *Invest. Ophthalmol. Vis. Sci.*, 42, 429–432.
- Luetscher, J. A., Kraemer, F. B., Wilson, D. M., Schwartz, H. C., & Bryer-Ash, M. (1985). Increased plasma inactive renin in diabetes mellitus. A marker of microvascular complications. *N. Engl. J. Med.*, 312, 1412–1417.
- Malhotra, R., Sadoshima, J., Brosius, F. C., 3rd, & Izumo, S. (1999). Mechanical stretch and angiotensin II differentially upregulate the renin–angiotensin system in cardiac myocytes in vitro. *Circ. Res.*, 85, 137–146.
- Miyajima, A., Kosaka, T., Asano, T., Asano, T., Seta, K., Kawai, T., et al. (2002). Angiotensin II type I antagonist prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis. *Cancer Res.*, 62, 4176–4179.
- Moravski, C. J., Kelly, D. J., Cooper, M. E., Gilbert, R. E., Bertram, J., Shahinfar, S., et al. (2000). Retinal neovascularization is prevented by blockade of the renin–angiotensin system. *Hypertension*, 36, 1099–1104.
- Moravski, C. J., Skinner, S. L., Stubbs, A. J., Sarlos, S., Kelly, D. J., Cooper, M. E., et al. (2003). The renin–angiotensin system influences ocular endothelial cell proliferation in diabetes: Transgenic and interventional studies. *Am. J. Pathol.*, 162, 151–160.
- Mullins, J. J., Peters, J., & Ganter, D. (1990). Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature*, 344, 541–544.
- Murata, T., Ishibashi, T., Khalil, A., Hata, Y., Yoshikawa, H., & Inomata, H. (1995). Vascular endothelial growth factor plays a role in hyperpermeability of diabetic retinal vessels. *Ophthal. Res.*, 27, 48–52.
- Nadal, J. A., Scigli, G. M., Carbini, L. A., Nussbaum, J. J., & Scigli, A. G. (1999). Angiotensin II and retinal pericytes migration. *Biochem. Biophys. Res. Commun.*, 266, 382–385.
- Nadal, J. A., Scigli, G. M., Carbini, L. A., & Scigli, A. G. (2002). Angiotensin II stimulates migration of retinal microvascular pericytes: Involvement of TGF- β and PDGF-BB. *Am. J. Physiol. Heart Circ. Physiol.*, 282, H739–H748.
- Nagai, N., Noda, K., Urano, T., Kubota, Y., Shinoda, H., Koto, T., et al. (2005). Selective suppression of pathologic, but not physiologic, retinal neovascularization by blocking the angiotensin II type 1 receptor. *Invest. Ophthalmol. Vis. Sci.*, 46, 1078–1084.

- Nagisa, Y., Shintani, A., & Nakagawa, S. (2001). The angiotensin II receptor antagonist candesartan cilexetil (TCV-116) ameliorates retinal disorders in rats. *Diabetologia*, *44*, 883–888.
- Otani, A., Takagi, H., Oh, H., Koyama, S., & Honda, Y. (2001). Angiotensin II induces expression of the Tie2 receptor ligand, angiopoietin-2, in bovine retinal endothelial cells. *Diabetes*, *50*, 867–875.
- Otani, A., Takagi, H., Oh, H., Suzuma, K., Matsumura, M., Ikeda, E., et al. (2000). Angiotensin II-stimulated vascular endothelial growth factor expression in bovine retinal pericytes. *Invest. Ophthalmol. Vis. Sci.*, *41*, 1192–1199.
- Otani, A., Takagi, H., Suzuma, K., & Honda, Y. (1998). Angiotensin II potentiates endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ. Res.*, *82*, 619–628.
- Paula, R. D., Lima, C. V., Khosla, M. C., & Santos, R. A. (1995). Angiotensin-(1–7) potentiates the hypotensive effect of bradykinin in conscious rats. *Hypertension*, *26*, 1154–1159.
- Penn, J. S., Tolman, B. L., & Lowery, L. A. (1993). Variable oxygen exposure causes preretinal neovascularization in the newborn rat. *Invest. Ophthalmol. Vis. Sci.*, *34*, 576–585.
- Pradhan, R., Fong, D., March, C., Jack, R., Rezapour, G., Norris, K., et al. (2002). Angiotensin-converting enzyme inhibition for the treatment of moderate to severe diabetic retinopathy in normotensive type 2 diabetic patients. A pilot study. *J. Diab. Complications*, *16*, 377–381.
- Provis, J. M., Leech, J., Diaz, C. M., Penfold, P. L., Stone, J., & Keshet, E. (1997). Development of the human retinal vasculature: Cellular relations and VEGF expression. *Exp. Eye Res.*, *65*, 555–568.
- Rong, P., Wilkinson-Berka, J. L., & Skinner, S. L. (1999). Renin in thymus, gut, hindlimb, and adrenal of (mRen-2)27 and normal rats: Secretion and content studies. *Am. J. Physiol.*, *277*, E639–E646.
- Rong, P., Wilkinson-Berka, J. L., & Skinner, S. L. (2001). Control of renin secretion from adrenal gland in transgenic Ren-2 and normal rats. *Mol. Cell Endocrinol.*, *173*, 203–212.
- Ruperez, M., Lorenzo, O., Blanco-Colio, L. M., Esteban, V., Egido, J., & Ruiz-Ortega, M. (2003). Connective tissue growth factor is a mediator of angiotensin II-induced fibrosis. *Circulation*, *108*, 1499–1505.
- Ruperez, M., Ruiz-Ortega, M., Esteban, V., Lorenzo, O., Mezzano, S., Plaza, J. J., et al. (2003). Angiotensin II increases connective tissue growth factor in the kidney. *Am. J. Pathol.*, *163*, 1937–1947.
- Sandercoe, T. M., Geller, S. F., Hendrickson, A. E., Stone, J., & Provis, J. M. (2003). VEGF expression by ganglion cells in central retina before formation of the foveal depression in monkey retina: Evidence of developmental hypoxia. *J. Comp. Neurol.*, *462*, 42–54.
- Sarlos, S., Rizkalla, B., Moravski, C. J., Cao, Z., Cooper, M. E., & Wilkinson-Berka, J. L. (2003). Retinal angiogenesis is mediated by an interaction between the angiotensin type 2 receptor, VEGF and angiopoietin. *Am. J. Pathol.*, *163*, 879–887.
- Sarlos, S., & Wilkinson-Berka, J. L. (2005). The renin–angiotensin system and the developing retinal vasculature. *Invest. Ophthalmol. Vis. Sci.*, *46*, 1069–1077.
- Sato, T., Niwa, M., Himeno, A., Tsutumi, K., & Amemiya, T. (1993). Quantitative receptor autoradiographic analysis for angiotensin II receptors in bovine retinal microvessels: Quantitation with radioluminography. *Cell Mol. Neurobiol.*, *13*, 233–245.
- Schonfelder, U., Hofer, A., Paul, M., & Funk, R. H. (1998). In situ observation of living pericytes in rat retinal capillaries. *Microvasc. Res.*, *56*, 22–29.
- Seko, Y., Fujikura, H., Pang, J., Tokoro, T., & Shimokawa, H. (1999). Induction of vascular endothelial growth factor after application of mechanical stress to retinal pigment epithelium of the rat in vitro. *Invest. Ophthalmol. Vis. Sci.*, *40*, 3287–3291.
- Sjolie, A. K., & Chaturvedi, N. (2002). The retinal renin–angiotensin system: Implications for therapy in diabetic retinopathy. *J. Hum. Hypertens.*, *16*(Suppl. 3), S42–S46.
- Smith, L. E., Wesolowski, E., McLellan, A., Kostyk, S. K., D'Amato, R., Sullivan, R., et al. (1994). Oxygen-induced retinopathy in the mouse. *Invest. Ophthalmol. Vis. Sci.*, *35*, 101–111.
- Song, K., Allen, A. M., Paxinos, G., & Mendelsohn, F. A. (1992). Mapping of angiotensin II receptor subtype heterogeneity in rat brain. *J. Comp. Neurol.*, *316*, 467–484.
- Stone, J., Itin, A., Alon, T., Peer, J., Gnessin, H., Chan-Ling, T., et al. (1995). Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J. Neurosci.*, *15*, 4738–4747.
- Su, E. N., Alder, V. A., Yu, D. Y., Yu, P. K., Cringle, S. J., & Yoganathan, K. (2000). Continued progression of retinopathy despite spontaneous recovery to normoglycemia in a long-term study of streptozotocin-induced diabetes in rats. *Graefes Arch. Clin. Exp. Ophthalmol.*, *238*, 163–173.
- Suzuki, J., Iwai, M., Nakagami, H., Wu, L., Chen, R., Sugaya, T., et al. (2002). Role of angiotensin II-regulated apoptosis through distinct AT1 and AT2 receptors in neointimal formation. *Circulation*, *106*, 847–853.
- Suzuki, Y., Ruiz-Ortega, M., Lorenzo, O., Ruperez, M., Esteban, V., & Egido, J. (2003). Inflammation and angiotensin II. *Int. J. Biochem. Cell Biol.*, *35*, 881–900.
- Suzuma, I., Hata, Y., Clermont, A., Pokras, F., Rook, S. L., Suzuma, K., et al. (2001). Cyclic stretch and hypertension induce retinal expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2: Potential mechanisms for exacerbation of diabetic retinopathy by hypertension. *Diabetes*, *50*, 444–454.
- Tadesse, M., Yan, Y., Yossuck, P., & Higgins, R. D. (2001). Captopril improves retinal neovascularization via endothelin-1. *Invest. Ophthalmol. Vis. Sci.*, *42*, 1867–1872.
- Tamura, K., Nyui, N., Tamura, N., Fujita, T., Kihara, M., Toya, Y., et al. (1998). Mechanism of angiotensin II-mediated regulation of fibronectin gene in rat vascular smooth muscle cells. *J. Biol. Chem.*, *273*, 26487–26496.
- Tikellis, C., Johnston, C. I., Forbes, J. M., Burns, W. C., Thomas, M. C., Lew, R. A., et al. (2004). Identification of angiotensin converting enzyme 2 in the rodent retina. *Curr. Eye Res.*, *29*, 419–427.
- Timmermanns, P. B. M. W. M., Chiu, A. T., Herblin, W. F., Wong, P. C., & Smith, R. D. (1992). Angiotensin II receptor subtypes. *Am. J. Hypertens.*, *5*, 406–410.

- Tout, S., Chan-Ling, T., Hollander, H., & Stone, J. (1993). The role of Muller cells in the formation of the blood–retinal barrier. *Neuroscience*, *55*, 291–301.
- Touyz, R. M., & Berry, C. (2002). Recent advances in angiotensin II signalling. *Braz. J. Med. Biol. Res.*, *35*, 1001–1015.
- UKPDS Study Group. (1998). Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 39. UK Prospective Diabetes Study Group. *BMJ*, *317*, 713–720.
- Volpert, O. V., Ward, W. F., Lingen, M. W., Chesler, L., Solt, D. B., Johnson, M. D., et al. (1996). Captopril inhibits angiogenesis and slows the growth of experimental tumors in rats. *J. Clin. Invest.*, *98*, 671–679.
- Wagner, J., Danser, A. H. J., Derkx, F. H. M., de Jong, P. T. V. M., Paul, M., Mullins, J. J., et al. (1996). Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: Evidence for an intraocular renin–angiotensin system. *Br. J. Ophthalmol.*, *80*, 159–163.
- Watkins, P. J. (2003). Retinopathy. *BMJ*, *326*, 924–926.
- Wheeler-Schilling, T. H., Kohler, K., Sautter, M., & Guenther, E. (1999). Angiotensin II receptor subtype gene expression and cellular localization in the retina and non-neuronal ocular tissues of the rat. *Eur. J. Neurosci.*, *11*, 3387–3394.
- Wilkinson-Berka, J. L., Kelly, D. J., Koerner, S. M., Jaworski, K., Davis, B., Thallas, V., et al. (2002). ALT-946 and aminoguanidine, inhibitors of advanced glycation, improve severe nephropathy in the diabetic transgenic (mREN-2)27 rat. *Diabetes*, *51*, 3283–3289.
- Wilkinson-Berka, J. L., Kelly, D. J., Rong, P., Campbell, D. J., & Skinner, S. L. (2002). Characterisation of a thymic renin–angiotensin system in the transgenic m(Ren-2)27 rat. *Mol. Cell Endocrinol.*, *194*, 201–209.
- Williams, B., Baker, A. Q., Gallacher, B., & Lodwick, D. (1995). Angiotensin II increases vascular permeability factor gene expression by human vascular smooth muscle cells. *Hypertension*, *25*, 913–917.
- Witmer, A. N., Blaauwgeers, H. G., Weich, H. A., Alitalo, K., Vrensen, G. F., & Schlingemann, R. O. (2002). Altered expression patterns of VEGF receptors in human diabetic retina and in experimental VEGF-induced retinopathy in monkey. *Invest. Ophthalmol. Vis. Sci.*, *43*, 849–857.
- Wong, T. Y., Hubbard, L. D., Klein, R., Marino, E. K., Kronmal, R., Sharrett, A. R., et al. (2002). Retinal microvascular abnormalities and blood pressure in older people: The Cardiovascular Health Study. *Br. J. Ophthalmol.*, *86*, 1007–1013.
- Yamagishi, S., Amano, S., Inagaki, Y., Okamoto, T., Inoue, H., Takeuchi, M., et al. (2003). Angiotensin II-type 1 receptor interaction upregulates vascular endothelial growth factor messenger RNA levels in retinal pericytes through intracellular reactive oxygen species generation. *Drugs Exp. Clin. Res.*, *29*, 75–80.
- Ye, M., Wysocki, J., Naaz, P., Salabat, M. R., LaPointe, M. S., & Batlle, D. (2004). Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: A renoprotective combination? *Hypertension*, *43*, 1120–1125.
- Yourey, P. A., Gohari, S., Su, J. L., & Alderson, R. F. (2000). Vascular endothelial cell growth factors promote the in vitro development of rat photoreceptor cells. *J. Neurosci.*, *20*, 6781–6788.
- Zhang, X., Lassila, M., Cooper, M. E., & Cao, Z. (2004). Retinal expression of vascular endothelial growth factor Is mediated by angiotensin type 1 and type 2 receptors. *Hypertension*, *43*, 276–281.
- Zhuo, J., Alcorn, D., McCausland, J., & Mendelsohn, F. A. (1994). Localization and regulation of angiotensin II receptors in renomedullary interstitial cells. *Kidney Int.*, *46*, 1483–1485.

Expert Opinion

1. Background
2. Medical need
3. Existing treatment
4. Market review
5. Current research goals and scientific rationale
6. Competitive environment
7. Potential developmental issues
8. Expert opinion

Emerging drugs which target the renin–angiotensin–aldosterone system

Ulrike Muscha Steckelings, Ludovit Paulis, Thomas Unger & Michael Bader[†]

[†]Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, Berlin, Germany

Introduction: The renin–angiotensin–aldosterone system (RAAS) is already the most important target for drugs in the cardiovascular system. However, still new developments are underway to interfere with the system on different levels.

Areas covered: The novel strategies to interfere with RAAS aim to reduce the synthesis of the two major RAAS effector hormones, angiotensin (Ang) II and aldosterone, or interfere with their receptors, AT1 and mineralocorticoid receptor, respectively. Moreover, novel targets have been identified in RAAS, such as the (pro)renin receptor, and molecules, which counteract the classical actions of Ang II and are therefore beneficial in cardiovascular diseases. These include the AT2 receptor and the ACE2/Ang-(1-7)/Mas axis. The search for drugs activating these tissue-protective arms of RAAS is therefore the most innovative field in RAAS pharmacology.

Expert opinion: Most of the novel pharmacological strategies to inhibit the classical RAAS need to prove their superiority above the existing treatment in clinical trials and then have to compete against these now quite cheap drugs in a competitive market. The newly discovered targets have functions beyond the cardiovascular system opening up novel therapeutic areas for drugs interfering with RAAS components.

Keywords: aldosterone, angiotensin, heart failure, hypertension

Expert Opin. Emerging Drugs (2011) 16(4):619-630

1. Background

The renin–angiotensin–aldosterone system (RAAS) is a major physiological regulator of blood pressure and blood volume [1-3]. In addition, it is also responsible for hypertrophic and fibrotic damage in cardiovascular end organs accompanying diseases such as hypertension and diabetes. The main effector molecules of RAAS are the peptide angiotensin (Ang) II and the steroid hormone aldosterone. Ang II is generated by a two-step proteolytic process from the precursor angiotensinogen first by renin releasing the decapeptide Ang I, which then is converted to the octapeptide Ang II by angiotensin-converting enzyme (ACE) (Figure 1) [1]. Ang II activates two seven-transmembrane receptors, AT1 and AT2. AT1 receptors confer most of the *classical* actions of Ang II, which include vasoconstriction, sodium retention in the kidney, sympathetic activation in the CNS and the release of aldosterone from the adrenal gland, which in turn also mediates sodium retention via the mineralocorticoid receptor (MR). In addition to these pro-hypertensive actions, Ang II and aldosterone also exert hypertrophic and profibrotic effects in heart, kidney and vessels. Aldosterone is synthesised in the glomerulosa cells of the adrenal gland from corticosterone by the enzyme 11-β-hydroxylase (CYP11B2), also called aldosterone synthase.

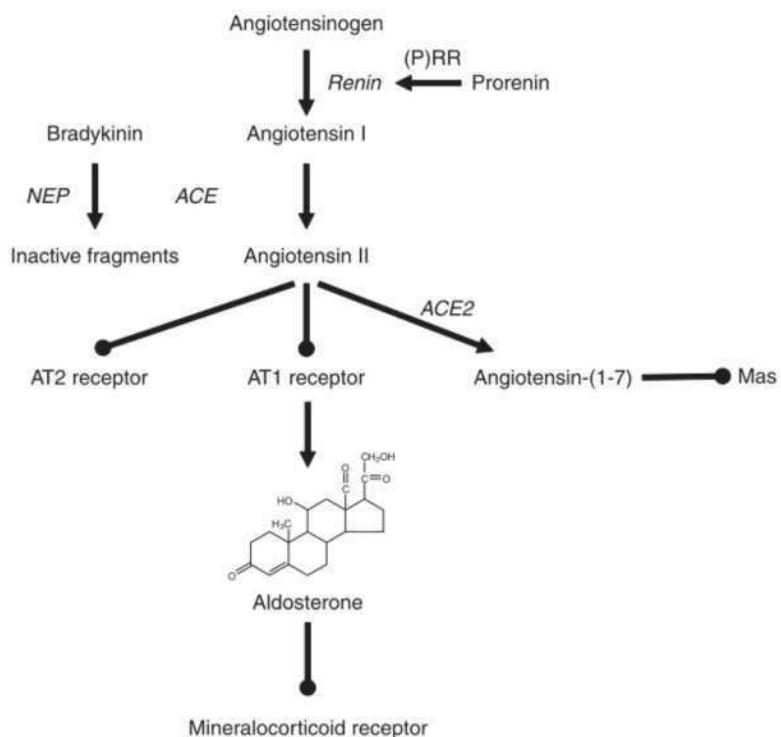


Figure 1. Scheme of the renin-angiotensin-aldosterone system (RAAS). Peptides are in black, enzymes in italics.

ACE: Angiotensin-converting enzyme; NEP: Neutral endopeptidase 24.11; (P)RR: (Pro)renin receptor; Mas: Mas receptor.

Ang II is, however, not only generated in the circulation but also locally in tissues, such as kidney, heart, brain and adrenal gland [2,3]. These local RAASs may be pathophysiological even more important than the systemic RAAS. A novel component of RAAS has recently been described which may be of special relevance in this respect. The (pro) renin receptor ((P)RR) binds prorenin and renin and activates it inside tissues [4-6]. Furthermore, this molecule induces several signalling pathways on renin binding which may contribute to the organ-damaging effects of RAAS. However, (P)RR has also been shown to interact with the vesicular proton pump and to exert actions completely independent of RAAS [6].

In contrast to the above-discussed RAAS components, which all contribute to the pathology of hypertension or end-organ damage, RAAS additionally harbours a ‘protective arm’ with active hormones (angiotensin-(1-7), Ang-(1-7)), enzymes (ACE2) and receptors (AT2 and Mas) mediating tissue-protective actions [7,8].

Ang-(1-7) is generated from Ang II by the carboxypeptidase ACE2 and interacts with its receptor Mas. Interestingly, this heptapeptide seems to counteract most of the *classical* actions of Ang II being antihypertensive, antihypertrophic, antifibrotic and improving the metabolic status [7].

Also the AT2 receptor, which is activated by Ang II, has been reported to interfere with AT1 receptor-coupled actions, but also with cytokines and growth factors thereby

acting anti-inflammatory, antiproliferative, antifibrotic, anti-apoptotic and neuroregenerative [8,9].

Pharmacological interference with RAAS is a standard therapeutic approach in cardiovascular disease with cardiovascular disease being the leading cause of death worldwide. Current treatments all aim at preventing the deleterious actions of Ang II mediated via the AT1 receptor. Therapeutic exploitation of the protective arm of RAAS is still in development.

2. Medical need

It is estimated that almost one billion people worldwide suffer from hypertension and it is expected that their number will rise by 60% within the coming 15 years [10]. Although several potent antihypertensives are already on the market, in only about 25% of patients the reduction of blood pressure meets levels recommended by current guidelines [11,12]. There are three main reasons responsible for this unsatisfactory situation: i) compliance of patients is insufficient. This problem may be addressed by developing more potent antihypertensive drugs with long duration of action which allow effective treatment with one pill once daily (or less); ii) awareness of physicians is unsatisfactory. This problem is addressed with increasing success by national and international educational programmes and iii) there is a certain percentage of patients in which hypertension cannot be

controlled with current treatments, not even with the combination of three to four antihypertensives. They are suffering from so-called 'resistant hypertension'.

Patients with uncontrolled hypertension have a reduced life expectancy and an increased risk of disability due to the development of hypertension-induced end-organ damage which involves cardiac, renal, cerebrovascular and ophthalmological disease. When combining end-organ damage due to hypertension and diabetes, 60 – 80% of all heart failure [13,14] and 70% of renal failure [15,16] cases can be attributed to these causes.

Furthermore, diabetes and hypertension are the major causes of blindness in patients of working age in industrialised countries [17-19]. Although current therapeutic strategies quite effectively lower blood pressure, their ability to additionally prevent end-organ damage is limited or absent [20,21]. Currently used RAAS-blocking drugs seem to represent antihypertensives with certain tissue-protective properties.

Consequently, according to current guidelines [22], ACE inhibitors and AT1-receptor blockers (ARBs) are first-line treatment for diabetic nephropathy no matter whether the patient is hypertensive or not [23]. However, effectiveness of these drugs is limited and on average end-stage renal disease and dialysis can only be delayed for a few months.

Taken together, although a broad selection of antihypertensives is already on the market, hypertension in a majority of patients is still not sufficiently controlled, a problem which may be improved by the development of antihypertensives with additional tissue-protective properties and with sufficient potency to be effective in patients with resistant hypertension.

3. Existing treatment

Currently, there are four drug classes approved for clinical use which pharmacologically interfere with RAAS: ACE inhibitors, ARBs, direct renin inhibitors (DRIs) and aldosterone antagonists [1,2]. All of these drugs aim at preventing the deleterious effects of excess Ang II and aldosterone mediated via the AT1 receptor and the MR, respectively. DRIs and ACE inhibitors function by interrupting Ang II synthesis through inhibition of the essential enzymes necessary for cleavage of angiotensinogen (renin) or Ang I (ACE), respectively. ARBs are specific antagonists for the AT1 receptor and compete with Ang II for binding to this receptor. Aldosterone antagonists block the binding of the hormone to the MR.

In addition to these classical RAAS-interfering drugs, β -blockers also interact with RAAS by inhibition of renin release from the juxtaglomerular apparatus thus diminishing Ang II synthesis.

3.1 ACE inhibitors

ACE inhibitors were originally isolated from the venome of the Brazilian snake *Bothrops jararaca* [24]. Captopril and the first follow-up product, enalapril (which already possessed a much longer plasma half-life than captopril), rapidly proved to be highly effective in lowering blood pressure together

with very good tolerability [25]. In terms of side effects, the major problem of ACE inhibitors is chronic cough, which leads to discontinuation of treatment in up to 20% of patients [26]. Moreover, ACE inhibitors cause angioedema in < 0.1% of patients, which – although very rare – is a potentially life-threatening complication [27]. Both characteristic side effects, cough and angioedema, are thought to be caused by accumulation of bradykinin and other kinins, which are cleaved and inactivated by ACE. Despite these side effects, the risk-benefit profile of ACE inhibitors is most favourable and, consequently, ACE inhibitors belong to first-line treatment for hypertension.

The HOPE (Heart Outcomes Prevention Evaluation) study in 2000 was a landmark study in the history of cardiovascular drug development showing that pharmacological interference with RAAS by ACE inhibition with ramipril reduced mortality in patients at cardiovascular risk more than would have been expected by the modest fall in blood pressure in the almost normotensive study population [28]. Despite a still ongoing discussion about the relative role of blood pressure reductions in this study, these results suggest an additional, non-blood pressure-related benefit of RAAS inhibitors beyond their pure antihypertensive action.

The success of treatment with ACE inhibitors is indicated by the multitude of ACE inhibitors currently on the market which comprise captopril, enalapril, lisinopril, perindopril, cilazapril, benazepril, quinapril, fosinopril, ramipril, moexipril and trandolapril.

3.2 AT1-receptor blockers

The first ARB, losartan, was approved for clinical treatment in 1995 [29,30]. Since ARBs display no interaction with kinin metabolism, it was hoped that the typical adverse reactions of ACE inhibitors, angioedema and chronic cough, would not occur in patients treated with ARBs. The incidence of chronic cough in patients treated with ARBs indeed turned out to be at placebo level, while angioedema still seems to occur, although at much lower rate than with ACE inhibitors [31].

ARBs have proven to be equally effective as ACE inhibitors in lowering blood pressure and reducing cardiovascular morbidity and mortality [32]. A series of large-scale clinical trials further showed effectiveness in chronic heart failure [33], diabetic kidney disease [34] and retinopathy [35]. Since ARBs (as ACE inhibitors) were superior to other antihypertensives in reducing end points such as death, myocardial infarction or stroke [36,37] despite an equal reduction in blood pressure, it is speculated that ARBs may have favourable features extending their antihypertensive effects [38]. Such features may be explained by reactively increased renin and Ang II levels [39,40], which in turn stimulate the unopposed AT2 receptors [41-43]. AT2 receptors have been described to oppose the AT1 receptor and act as tissue-protective agents [9]. In animal experiments, blockade of AT2 receptors in fact abolishes favourable effects of ARBs, for example, in the context of stroke prevention, vasodilation or kidney fibrosis [41-44].

A subgroup of ARBs (telmisartan, irbesartan and to a lesser extent losartan) have been shown to have peroxisome proliferator-activated receptor-gamma (PPAR γ)-agonistic properties resulting in an improvement of glucose and lipid metabolism [45]. Interestingly, PPAR γ -agonistic ARBs apparently do not suffer from typical side effects of classical PPAR γ agonists, the glitazones, such as weight gain, oedema or even increased cardiovascular complications. On the other hand, however, the clinical relevance of the PPAR γ -modulating activity of some ARBs is still not really clear.

Several ARBs (losartan, telmisartan, candesartan, valsartan, eprosartan, irbesartan, olmesartan, zolasartan) are currently on the market in Europe and the USA for monotherapy or in combination with other antihypertensives, preferably with diuretics and calcium channel blockers.

3.3 Direct renin inhibitors

Direct renin inhibition seems the most logical and straightforward way of inhibiting RAAS and has already been suggested as such by one of the pioneers of RAAS research, Leonard Skeggs, who wrote in 1957 ‘Since renin is the initial and rate-limiting substance in the renin-hypertensin system it would seem that this last approach (i.e., inhibition of renin) would be the most likely to succeed’ [46]. Consequently, direct renin inhibitors (DRIs) have been synthesised and tested for many years, but all of them turned out to have a very poor oral bioavailability and potentially high production costs which prevented further development. Only recently, a first renin inhibitor, aliskiren, has been approved for the treatment of hypertension [47]. Aliskiren is currently tested in several large-scale clinical trials for tissue-protective properties beyond its antihypertensive effect [48].

3.4 Aldosterone receptor antagonists

The first aldosterone receptor blocker, spironolactone, has been marketed since the early 60s, as such being the first drug directly targeting RAAS. The beneficial effects of spironolactone are attributed to the blockade of the MR in the kidneys and non-epithelial tissues such as the myocardium, brain or blood vessels [49]. The MR is responsible for aldosterone effects such as sodium and water retention, myocardial fibrosis or vasoconstriction. Most of these effects are ‘slow’ genomic effects triggered by aldosterone binding to its cytosolic receptors, but some of them are to yet unknown extent mediated by ‘fast’ non-genomic effects [50]. Spironolactone was indicated for hyperaldosteronism, hypokalaemia, oedematous states (including congestive heart failure) and essential hypertension [51]. It was shown to effectively reduce blood pressure [52]. Although aldosterone is a downstream effector of RAAS, the introduction of ACE inhibitors and ARBs did not mean the dawn of aldosterone blockade. It was observed that after temporary reduction, the aldosterone levels are restored or even elevated following RAAS inhibition [53]. This phenomenon of aldosterone escape provided the rationale for dual RAAS blockade with an ACE inhibitor and aldosterone receptor

antagonist. In RALES (Randomized Aldosterone Evaluation Study), the addition of spironolactone to conventional therapy (including ACE inhibition) further markedly reduced morbidity and mortality in patients with severe heart failure [54]. However, the use of spironolactone is limited by its low MR selectivity leading to adverse progesterone- and testosterone-dependent side effects, such as gynaecomastia, breast pain, menstrual irregularities and loss of libido. Since 2002, a novel aldosterone receptor blocker called eplerenone is available. In EPHESUS (Eplerenone Heart Failure Efficacy and Survival Study), a study with similar design to RALES, eplerenone reduced morbidity and mortality in heart failure patients when added to standard medication [55]. Similarly to spironolactone, eplerenone effectively lowered blood pressure compared with [56–58] or even when added to [59] conventional therapy. However, in contrast to spironolactone, it has 100 – 1000 lower affinity to testosterone and progesterone receptors, meaning less pronounced sexual side effects [60]. Although both spironolactone and eplerenone are associated with hyperkalaemia [60], the shorter half-life of eplerenone could ease the restoration of normal potassium levels [61]. Compared with spironolactone, treatment with eplerenone is not associated with increased haemoglobin A1c (HbA1c) levels [62], an independent risk factor in heart failure patients. On the other hand, eplerenone affinity to MR is 20 times lower compared with spironolactone *in vitro* [61] and higher eplerenone doses are required to achieve the same blood pressure reducing effect as with spironolactone [63].

4. Market review

Cardiovascular and metabolic disease drugs including antihypertensives represent a huge medical market. In 2009, the market of antihypertensives amounted to \$27.2 billion with an annual growth rate of 5% since 2002. However, growth rate is expected to slow down to about 1.5% per year to reach a market volume of \$30 billion in 2016 (Source: GBI Research, <http://www.articlesnatch.com/Article/Antihypertensives-Market-To-2016/1928476>). This decline in growth is due to generic erosion, in particular of RAAS drugs. The best selling substances are the ARBs losartan and valsartan; both of which lose patent protection, losartan lost it already in 2010 and valsartan will lose it in 2011 in Europe and in 2012 in the USA.

Unmet medical needs still exist but they will be mainly met by improvements of current therapeutic principles, for example, by the marketing of drug combinations, rather than by new original drugs to treat hitherto untreated patient populations.

5. Current research goals and scientific rationale

The research and development of novel compounds interfering with RAAS has mainly four goals. They are mentioned in Sections 5.1 – 5.4.

5.1 Novel compounds of the already established classes and combination drugs

Novel ARBs, DRIs and aldosterone antagonists are in the pipeline of pharmaceutical companies. But more importantly, these drugs are developed and already marketed in fixed combinations with each other or with drugs of other classes, such as statins, β -blockers, diuretics or calcium channel blockers (<http://www.phrma.org/research/heart-disease-stroke>). These combinations will not be discussed in this review.

5.2 Compounds in these classes but carrying additional functions

Substances have been developed, which are ACE inhibitors or ARBs but at the same time inhibit other proteases, block other receptors or release nitric oxide (NO). The goal is to get drugs with synergistic double actions and allowing a lower dosing. However, side effects may be potentiated by combining two synergistic actions in one compound.

5.3 Compounds directed towards novel targets within RAAS

Novel RAAS targets are the (P)RR receptor and the components of the beneficial arm of RAAS, ACE2, Ang-(1-7), Mas and the AT2 receptor. For all these factors drugs are at different stages of development. Furthermore, a first example of a novel kind of drug is tested in the context of RAAS, which are biased agonists of G-protein coupled receptors. These molecules are activators of receptors, in this case of the AT1 receptor, but only stimulate selective signalling pathways, in this case the β -arrestin signalling pathway and thereby the beneficial action of Ang II [64,65].

5.4 Non-classical pharmaceutical approaches

Two novel approaches fall into this class, the vaccination against angiotensin peptides and renal nerve ablation. Both have the disadvantage to be irreversible or at least very long-lasting (vaccination), however, renal nerve ablation has been proven to be very effective in lowering blood pressure.

6. Competitive environment

6.1 Vasopeptidase inhibitors

As mentioned above, ACE inhibitors not only inhibit the generation of Ang II but also the degradation of kinins, which may be an additional part of their therapeutic effect, since kinins are known to exert protective actions in cardiovascular organs [66]. Furthermore, natriuretic peptides are beneficial for the cardiovascular system, since they induce sodium excretion, vasodilation and antihypertrophic effects in the heart [67]. The enzyme neutral endopeptidase 24.11 (NEP) degrades kinins and natriuretic peptides [68]. Therefore, compounds were developed, which show a combined inhibitory effect on ACE and NEP, the vasopeptidase inhibitors omapatrilat, fosinoprilat and sampatrilat [69]. First clinical trials proved their

effectiveness, however, they also revealed an aggravated side effect profile compared with ACE inhibitors [68]. In particular, there was an unacceptably high incidence of potentially life-threatening angioedemas in African Americans, which was probably due to the increased accumulation of kinins. As a consequence, these drugs were not developed further.

Anyhow, the concept of dual inhibition of vasopeptidases has been pursued, however, sparing NEP inhibition. Combined inhibitors were developed targeting ACE and ECE-1, the endothelin-converting enzyme, which activates endothelins [70]. Endothelins are strongly vasoconstricting peptides by acting on their ETA receptor [71]. Some of these substances show an interesting specificity for only one of the two active sites of ACE, the C-domain, which is mainly responsible for the generation of Ang II, while the N-domain remains relatively unaffected leaving other functions of ACE such as bradykinin degradation intact and reducing the risk of side effects [72]. These substances have shown blood pressure lowering actions in hypertensive rats [70] but have not yet entered clinical trials.

6.2 AT1 receptor interacting compounds

AT1 receptor blockade is still an important target for drugs newly developed by the pharmaceutical industry. Two new pure AT1 blockers and one novel compound with additional actions are in Phase III clinical trials. The two classical AT1 blockers are fimasartan from Boryung (Seoul, South Korea) and azilsartan from Takeda (Osaka, Japan) and they are developed for hypertension (Table 1).

LCZ696 from Novartis (Basel, Switzerland) not only antagonises AT1 but also blocks NEP. The stabilisation of cardioprotective kinins and natriuretic peptides by NEP inhibition contributes to the protective actions of an AT1 antagonist [73]. LCZ696, as a prototypic substance for the new class of ARNIs (AT1 receptor and NEP inhibitors), has been compared with a classical AT1 antagonist in a recent clinical trial and has shown higher efficiency in blood pressure reduction without additional adverse effects [74], which may have been expected based on the experiences with combined ACE/NEP inhibitors. Nevertheless, NEP inhibition remains problematic, since it has been shown that NEP is responsible for the degradation of amyloid peptides in Alzheimer's disease [75] and since NEP-deficient mice become obese at higher age, the reason for which is unknown [76]. Thus, severe long-term side effects of NEP inhibition cannot be ruled out.

Furthermore, there is another compound now in Phase II clinical trials which binds to the AT1 receptor, however, not only inhibiting it, but also activating some of its signalling functions [64]. TRV-120027 from Trevena (King of Prussia, USA) is the first example of a biased agonist for a G-protein coupled receptor which has entered clinical trials (<http://www.trevenainc.com/news-details.php?id=25>). Its action is based on the stabilisation of a certain conformation of

Emerging drugs which target the renin–angiotensin–aldosterone system

Table 1. Competitive environment table.

Compound	Company	Indication	Stage of development	Mechanism of action
Fimasartan	Boryung	Hypertension	Phase III ongoing	AT1 antagonist
Azilsartan	Takeda	Hypertension	Phase III ongoing	AT1 antagonist
LCZ696	Novartis	Hypertension, Heart failure	Phase III ongoing	ARNI
PS433540	Ligand Pharmaceuticals	Hypertension	Phase II completed	AT1 and ETA antagonist (DARA)
TRV-120027	Trevena	Heart failure, CNS	Phase II ongoing	β -Arrestin-biased AT1 ligand
LCI699	Novartis	Hypertension	Phase II ongoing	Aldosterone synthase inhibitor
VTP-27999	Vitae Pharmaceuticals	Chronic kidney diseases	Phase I completed	Renin inhibitor
APN01	Apeiron/GSK	Heart failure, Acute respiratory distress syndrome	Phase I completed	Recombinant ACE2
ATV	BTG International	Hypertension	Phase II ongoing	Vaccine against Ang I
CYT006-AngQb	Cytos Biotechnology	Hypertension	Phase II ongoing	Vaccine against Ang II

ACE: Angiotensin-converting enzyme; Ang: Angiotensin; ARNI: AT1 receptor and NEP inhibitors; ATV: Angiotensin therapeutic vaccine; CNS: Central nervous system; DARA: Dual-acting receptor agonist; ETA: Endothelin receptor ETA; NEP: Neutral endopeptidase 24.11.

the AT1 receptor which only allows signalling through β -arrestin and the internalisation and degradation of the receptor. In animal experiments, TRV-120027 reduced mean arterial pressure, as did the unbiased AT1 antagonists, but, unlike those molecules, which decreased cardiac performance, it increased cardiac performance [64]. These effects were explained by a sequestration of the AT1 receptor reducing blood pressure but at the same time by an activation of the extracellular signal-regulated kinases (ERK) 1/2 and NO signalling pathways increasing cardiac stroke volume.

6.3 Dual AT1 and ETA receptor antagonists

A combined AT1 and ETA antagonist, PS433540, was developed by Pharmacopeia, a company later taken over by Ligand Pharmaceuticals (La Jolla, USA). This first dual-acting receptor agonist (DARA) inhibiting two major vasoconstricting peptide systems successfully passed a Phase II clinical trial for hypertension (http://www.drugs.com/clinical_trials/ligand-announces-phase-iib-results-dara-6677.html) in 2009, but anyhow seems to not be developed further (<http://www.ligand.com/business-development-opportunities>).

6.4 NO releasing AT1 antagonists and ACE inhibitors

Another attempt to increase potency of ARBs is the combination of an approved ARB with NO-releasing entities in one molecule [77]. The French company NicOx is designing a whole portfolio of compounds which all follow the principle of combining an established drug (such as cyclooxygenase (COX) inhibitors, statins or ARBs/ACE inhibitors) with an NO-releasing structure. NO is known to act vasodilatory thus lowering blood pressure. In addition, it also has tissue-protective properties. Both features may be additive to similar actions of ARBs/ACE inhibitors thus potentiating their blood-pressure lowering effects and also tissue-protective actions beyond the pure antihypertensive effect. NO-releasing ARBs/ACE inhibitors originally designed and synthesised by

NicOx, are now being developed by Merck and have entered Phase I clinical evaluation (<http://www.pharma.org/research/heart-disease-stroke>).

6.5 Direct renin inhibitors

Renin inhibition as most rational way of RAAS inhibition has not been pharmacologically exploited very much. Just one drug has entered the market, aliskiren from Novartis (Basel, Switzerland). All previous attempts have been hampered by the fact that DRIs all seem to have a very low oral bioavailability (oral bioavailability of aliskiren is 2–3%). A DRI developed by Actelion together with Merck was not successful in Phase II trials and the development was therefore stopped, but other DRIs are still in the pipeline (<http://www.actelion.com/en/journalists/medieninformationen.page?newsId=1261098>).

One other substance, VTP-27999 from Vitae Pharmaceuticals (Fort Washington, USA), has just successfully completed a Phase I clinical trial and a Phase IIb trial is planned for the end of 2011 (<http://www.vitapharma.com/view.cfm/56/Chronic-Kidney-Disease-Renin>). However, indication is not hypertension as for aliskiren, but chronic kidney diseases induced by diabetes or hypertension.

6.6 (P)RR antagonists

It is now well accepted that renin not only cleaves angiotensinogen thus generating Ang I, but it also binds to a high-affinity receptor, the so-called (P)RR [78]. As suggested by the terminology, (P)RR not only binds renin, but also the precursor, prorenin which is converted into its enzymatically active form on binding. Furthermore, binding of renin or prorenin to (P)RR elicits several signalling cascades such as activation of ERK 1/2 or translocation of the transcription factor PLZF (promyelocytic leukaemia zinc finger) [4,79]. Functionally, (P)RR activation seems to essentially contribute to the development of diabetic end-organ damage [80]. Intriguingly, in experimental diabetic nephropathy, blockade

of (P)RR not only prevented development but also reversed kidney damage [81]. Blockade of (P)RR in these experiments was obtained with a synthetic peptide containing the amino acid sequence corresponding to the 'handle' region of the pro-renin prosegment thus preventing prorenin from binding to the (P)RR and getting enzymatically activated. Being a peptide with very limited *in vivo* stability, the handle region peptide may be developed as an injectable drug if at all. However, it would be more favourable to develop a classical, small-molecule (P)RR antagonist with oral bioavailability. Such a developmental programme is currently being performed by CCR-Pharma Therapeutics, a spin-off company of Charité, Berlin [82].

6.7 Aldosterone antagonists and synthase inhibitors

Despite the better tolerability of eplerenone, it still shows some androgen receptor-related adverse events and its effectiveness compared with spironolactone is still questioned. Therefore, there is a search for novel aldosterone antagonising agents with similar potency as spironolactone and even higher specificity than eplerenone such as SM-368229 from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan) [83].

A novel approach is to move from receptor blockade to the inhibition of aldosterone synthesis. At least three compounds with this mechanism of action were identified: FAD286 (Novartis, Basel, Switzerland), LCI699 (Novartis, Basel, Switzerland) and SPP2745 (Novartis, Basel, Switzerland) [84]. FAD286, an enantiomer of fadrozol with an inhibitory effect on aldosterone synthase, reduced blood pressure and attenuated myocardial and renal target-organ damage [85,86] and normalised redox status in rats after myocardial infarction [87]. SPP2745 suppressed aldosterone levels and also provided cardio-, reno- and vasculo-protective effects even when in combination with conventional therapy [84]. Finally, LCI699 suppressed aldosterone levels and lowered blood pressure by 4.1 mm Hg in 14 hypertensive patients, but also latently inhibited cortisone formation [88].

The concept of aldosterone synthase inhibition should prevent reactive increase in aldosterone levels and adverse androgen receptor-related effects, because the substances in development do not contain a steroid structure. On the other hand, MRs are also stimulated by cortisol and other ligands, that are released in conditions of augmented oxidative load [2,89]. Aldosterone synthase inhibitors will not oppose these aldosterone-independent mechanisms that might be blocked by spironolactone or eplerenone. Future studies will show what clinical implications these theoretical concerns will have.

6.8 Vaccination

It has been an intriguing idea for many years that activity of RAAS can be chronically downregulated through elimination of critical components of RAAS by vaccination against these respective components [90]. A major incentive for such an approach was to overcome the problem of non-compliance

conceiving that hypertensive patients (the majority of whom does not suffer from any symptoms) may rather adhere to two vaccinations per year than to daily oral medication.

Two RAAS-directed vaccinations are currently under development. Cytos Biotechnology (Schlieren, Switzerland) has tested a conjugate vaccine, CYT006-AngQb composed of Ang II chemically linked to recombinant virus-like particles derived from an RNA phage. CYT006-AngQb underwent a successful combined Phase I and Phase II study performed in 2005/2006 [91]. The Phase II study arm revealed that the highest dose of CYT006-AngQb significantly lowered mean ambulatory daytime blood pressure at week 14 (-9.0/-4.0 mm Hg) when compared with placebo. Blood pressure reduction by CYT006-AngQb was most evident in early morning hours. Vaccination was well tolerated and no serious adverse events were attributable to treatment with CYT006-AngQb. However, renin levels were only mildly elevated after vaccination which contrasts with much more pronounced increases in plasma renin after 'conventional' RAAS blockade and points to incomplete inhibition of the Ang II-AT1 axis [90,91]. In two subsequent Phase II studies, in which CYT006-AngQb was injected more frequently and with shorter intervals to obtain higher antibody titres, blood pressure reduction by vaccination was only marginal and did not reach statistical significance when compared with placebo (<http://www.pharma.org/research/heart-disease-stroke>). Cytos Biotechnology declared in a press release that it will continue development of CYT006-AngQb for hypertension, however, it is not clear how and when next steps will be taken (http://www.cytos.com/userfiles/file/Cytos_Press_E_091110.pdf).

A second antihypertensive, RAAS-based vaccine termed ATV (angiotensin therapeutic vaccine) consists of an Ang I peptide conjugated to carrier protein KLH (keyhole limpet haemocyanin) and is developed by BTG International (London, UK) [90]. An earlier version of this vaccine underwent a successful Phase I study and lowered blood pressure in salt-depleted healthy volunteers but not in hypertensive patients. A first Phase IIa study has apparently been performed as well but no detailed data have been published. BTG International seems to continue development of ATV for long-term control of hypertension (<http://www.brgplc.com/development/our-pipeline>).

6.9 ACE2/Ang-(1-7)/Mas agonists

The first compound, which has entered clinical trials and interacts with the beneficial arm of RAAS, the ACE2/Ang-(1-7)/Mas axis, is APN01 developed by Apeiron (Vienna, Austria) together with GlaxoSmithKline (<http://www.apeiron-biologics.com>). APN01 is recombinant human ACE2 and is indicated for heart failure and additionally for acute respiratory distress syndrome. It has been shown, that Ang II is detrimental and Ang-(1-7) is beneficial in acute lung diseases [92,93]. Since ACE2 destroys Ang II and generates Ang-(1-7), it became a novel therapeutic principle for these diseases. APN01 has just successfully completed Phase I

clinical trials and should enter Phase II trials at the end of 2011 (<http://www.apeiron-biologics.com>).

Moreover, the ACE2/Ang-(1-7)/Mas axis is currently being targeted in numerous preclinical studies. ACE2 activators [94], Mas agonists (e.g., by Compugen (http://www.cgen.com/Content.aspx?Page=CGEN_856_CGEN_857)) and an oral formulation of the Ang-(1-7) peptide [95] have been developed and have shown beneficial effects in animal models of hypertension and diabetes [94,96–98]. Aventis also had a programme on Mas agonists and has discovered active compounds (e.g., AVE0991), which however have been given up [99].

6.10 AT2 agonists

In analogy to Ang 1-7/Mas, actions mediated by the AT2 receptor are also opposing those of the AT1 receptor [9]. However, the AT2 receptor does not have a specific ligand, but binds Ang II with basically the same affinity as the AT1 receptor. Thus, in order to make use of the favourable effects coupled to the AT2 receptor, a specific AT2 agonist is needed. Such an agonist with drug-like properties has been designed and synthesised in 2004 and is currently in preclinical development showing tissue-protective features in various disease models [100,101]. The patent holder, Vicore Pharma, is planning a Phase I study in early 2012, but has not decided about a clinical indication yet.

6.11 Renal denervation

Recently, a non-pharmacological treatment of patients with therapy-resistant hypertension has been successfully tested in a randomized controlled clinical trial, the denervation of both kidneys using catheters [102]. One important effect of this surgical intervention is the blunting of the sympathetic activation of RAAS by induction of renin release from juxtaglomerular cells, which may be a major pathomechanism in hypertension. This intervention only leads to a partial denervation of the efferent renal fibre and should be a safe procedure since kidney transplantation is even accompanied by a total renal denervation without major long-term problems. The method will now be broadly tested in additional trials internationally and, thus, soon its general applicability can be evaluated.

7. Potential developmental issues

All RAAS interfering drugs currently used in clinical practise have an exceptionally low rate of side effects, the only significant limitation being the teratogenic potential of all RAAS-blocking drugs which has led to a Category C warning (risks cannot be ruled out) for the first trimester of pregnancy and a Category D warning (positive evidence of risk) for the second and third trimester. Since all new developments in this field have similar mechanisms of action as the existing drugs, no major developmental issues are to be expected except for contraindications in pregnancy. However, drugs which in addition to RAAS components also inhibit NEP may be

problematic since this enzyme has numerous other substrates and, thus, its inhibition did already cause unwanted side effects and more may emerge during long-term treatment, as already discussed above.

Exceptions may be the vaccines, which could bear a danger of allergic reactions, or the (P)RR antagonist, since the (P)RR has been reported to be a subunit of the ATPase, which acidifies intracellular vesicles, and to be involved in different cellular signalling events. Probably therefore, (P)RR deficiency is fatally lethal in mice [6].

8. Expert opinion

RAAS remains the most important drug target for cardiovascular diseases. The already marketed drugs, ACE inhibitors, ARBs and DRIs are equally effective in lowering blood pressure and show a very low side effect profile. Since most ACE inhibitors and some ARBs have already lost their patent protection and are now marketed as generics, it will become increasingly difficult to market novel drugs of these classes. In particular, DRIs will have to present with additional benefits to allow their future success in the market. The novel drugs with anti-RAAS activity and additional functions, such as NEP inhibition, ETA antagonism and NO release, have to show such benefit in clinical trials as well, but according to preclinical data there is a good chance that they will fulfil these criteria and succeed in the competitive market. Aldosterone antagonists with improved adverse effect profile have already demonstrated additional benefits in particular for the treatment of heart failure, but current molecules still lack optimal specificity and efficiency. Drugs inhibiting aldosterone synthesis may have the same beneficial effects, however, theoretically other steroids may still activate the MR and nullify their effect in the tissue. Only further research will show whether the concept of these drugs is valid.

Also novel ways to inhibit RAAS more permanently such as vaccination and renal nerve ablation may be successful on the market in particular for patients with resistant hypertension or in patients with insufficient drug compliance. However, these treatments need to prove their safety in a clinical setting, since their effects are long-lasting or – in the case of nerve ablation – even irreversible and therefore in both cases hard to control.

The novel targets in RAAS, such as ACE2, Mas, Ang-(1-7), AT2 and (P)RR, besides also having cardiovascular applications in particular in organ protection, may open completely new fields for RAAS drugs, such as lung diseases (ACE2) and regenerative medicine or chronic inflammatory disease (AT2).

Even ‘good, old’ losartan is currently tested for novel, unconventional indications, in particular for the prevention of aortic dilatation in Marfan syndrome [103]. Interestingly, latest data indicate that the therapeutic effect of AT1-receptor blockade in this condition may in fact be due to indirect

stimulation of the unopposed AT2 receptor by reactively elevated levels of Ang II [104], suggesting that the future drug class of AT2 agonists may become another therapeutic approach to treat aortic dilatation. There are more very promising preclinical results for the use of drugs which stimulate the protective arm of RAAS and clinical trials for some targets are already on the way. The outcome of these trials will be pivotal for the chance of RAAS drugs to go beyond cardiovascular borders.

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

1. Steckelings UM, Unger T. The renin-angiotensin-aldosterone system. Manual of Hypertension of the European Society of Hypertension. Informa Healthcare; London: 2008. p. 110-16
2. Bader M. Tissue renin-angiotensin-aldosterone systems: targets for pharmacological therapy. *Annu Rev Pharmacol Toxicol* 2010;50:439-65
- 3. Paul M, Poyan MA, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev* 2006;16:747-803
4. Nguyen G, Delarue F, Burckle C, et al. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 2002;109:1417-27
5. Burckle C, Bader M. Prorenin and its ancient receptor. *Hypertension* 2006;48:549-51
6. Sihl G, Rousselle A, Vil'ianovich L, et al. Physiology of the (pro)renin receptor: wnt of change? *Kidney Int* 2010;78:246-56
7. Alenina N, Xu P, Rentzsch B, Bader M. Genetically altered animal models for mas and angiotensin-(1-7). *Exp Physiol* 2008;93:5328-537
8. Steckelings UM, Rompe F, Kaschina E, et al. The past, present and future of angiotensin II type 2 receptor stimulation. *J Renin Angiotensin Aldosterone Syst* 2010;11:67-73
- 9. Steckelings UM, Kaschina E, Unger T. The AT2 receptor – a matter of love and hate. *Peptides* 2005;26:1401-9
10. Kearney PM, Whelton M, Reynolds K, et al. Global burden of hypertension:
- analysis of worldwide data. *Lancet* 2005;365:217-23
11. Bramlage P, Bohm M, Volpe M, et al. A global perspective on blood pressure treatment and control in a referred cohort of hypertensive patients. *J Clin Hypertens (Greenwich)* 2010;12:666-77
12. Kotchen TA. Hypertension control: trends, approaches, and goals. *Hypertension* 2007;49:19-20
13. Kannel WB. Incidence and epidemiology of heart failure. *Heart Fail Rev* 2000;5:167-73
14. Redon J. Antihypertensive treatment: should it be titrated to blood pressure reduction or to target organ damage regression? *Curr Opin Nephrol Hypertens* 2005;14:448-52
15. Atkins RC. The epidemiology of chronic kidney disease.. *Kidney Int Suppl* 2005;94:S14-18
16. Agodoa LY, Jones CA, Held PJ. End-stage renal disease in the USA: data from the United States Renal Data System. *Am J Nephrol* 1996;16:7-16
17. Girach A, Manner D, Porta M. Diabetic microvascular complications: can patients at risk be identified? A review. *Int J Clin Pract* 2006;60:1471-83
18. Lightman S, Towler HM. Diabetic retinopathy. *Clin Cornerstone* 2003;5:12-21
19. Buch H, Vinding T, Nielsen NV. Prevalence and causes of visual impairment according to World Health Organization and United States criteria in an aged, urban Scandinavian population: the Copenhagen City Eye Study. *Ophthalmology* 2001;108:2347-57
20. Banerjee D, Materson BJ. Blood pressure-independent impact of antihypertensive agents on cardiovascular and renal disease. *Curr Hypertens Rep* 2002;4:445-52
21. Sleight P. Angiotensin II and trials of cardiovascular outcomes. *Am J Cardiol* 2002;89:11A-6A
22. European Society of Hypertension-European Society of Cardiology guidelines committee. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens* 2003;21:1011-53
- 23. Mancia G, De Backer G, Dominiczak A, et al. 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens* 2007;25:1105-87
- 24. Ferreira SH. A bradykinin-potentiating factor (BPF) present in the venom of Bothrops jararaca. *Br J Pharmacol* 1965;24:163-9
- 25. Stamler JF, Brody MJ, Phillips MI. The central and peripheral effects of captopril (SQ 14225) on the arterial pressure of the spontaneously hypertensive rat. *Brain Res* 1980;186:449-503
26. Bangalore S, Kumar S, Messerli FH. Angiotensin-converting enzyme inhibitor associated cough: deceptive information from the Physicians' Desk Reference. *Am J Med* 2010;123:1016-30
27. Kaplan AP, Greaves MW. Angioedema. *J Am Acad Dermatol* 2005;53:373-88
28. Sleight P, Yusuf S, Pogue J, et al. Blood-pressure reduction and cardiovascular risk in HOPE study. *Lancet* 2001;358:2130-1
- 29. Landmark study for the efficiency of ACE inhibitors.

Declaration of interest

T Unger has received financial support or owned personal investments in the following categories during the past years: i) as grant of research support: Actelion, Bayer, Boehringer Ingelheim, Novartis, Sanofi-Aventis and ii) as a consultant, advisory board member, on the speaker's bureau or at sponsored speaking events: Actelion, Bayer, Boehringer Ingelheim, MSD, Novartis, Pfizer, Takeda, Vicore Pharma.

Emerging drugs which target the renin–angiotensin–aldosterone system

29. Angiotensin-receptor blocker approved as antihypertensive. *Am J Health Syst Pharm* 1995;52:1368
30. Wong PC, Barnes TB, Chiu AT, et al. Losartan (DuP 753), an orally active nonpeptide angiotensin II receptor antagonist. *Cardiovasc Drug Rev* 1991;9:317-39
- **First description of ARB.**
31. Yusuf S, Teo K, Anderson C, et al. Effects of the angiotensin-receptor blocker telmisartan on cardiovascular events in high-risk patients intolerant to angiotensin-converting enzyme inhibitors: a randomised controlled trial. *Lancet* 2008;372:1174-83
32. Vidy DG. Telmisartan, ramipril, or both in patients at high risk for vascular events. *Curr Hypertens Rep* 2008;10:343-4
33. McMurray JJ. Angiotensin inhibition in heart failure. *J Renin Angiotensin Aldosterone Syst* 2004;5(Suppl 1):S17-22
34. Laverman GD, Remuzzi G, Ruggenenti P. ACE inhibition versus angiotensin receptor blockade: which is better for renal and cardiovascular protection? *J Am Soc Nephrol* 2004;15(Suppl 1):S64-70
35. Sjolie AK, Dodson P, Hobbs FR. Does renin-angiotensin system blockade have a role in preventing diabetic retinopathy? A clinical review. *Int J Clin Pract* 2011;65:148-53
36. Lindholm LH, Ibsen H, Dahlöf B, et al. Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* 2002;359:1004-10
37. Dahlöf B, Devereux RB, Kjeldsen SE, et al. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet* 2002;359:995-1003
38. Bakris G. Are there effects of renin-angiotensin system antagonists beyond blood pressure control? *Am J Cardiol* 2010;105:21A-9A
39. Bader M, Ganter D. Regulation of renin. *J Mol Med* 2000;78:130-9
40. Kurtz A. Renin release: sites, mechanisms, and control. *Annu Rev Physiol* 2011;73:377-99
41. Habashi JP, Doyle JJ, Holm TM, et al. Angiotensin II type 2 receptor signaling attenuates aortic aneurysm in mice through ERK antagonism. *Science* 2011;332:361-5
42. Li J, Culman J, Hortnagl H, et al. Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. *FASEB J* 2005;19:617-19
43. Naito T, Ma LJ, Yang H, et al. Angiotensin type 2 receptor actions contribute to angiotensin type 1 receptor blocker effects on kidney fibrosis. *Am J Physiol Renal Physiol* 2010;298:F683-91
44. Duke LM, Evans RG, Widdop RE. AT2 receptors contribute to acute blood pressure-lowering and vasodilator effects of AT1 receptor antagonism in conscious normotensive but not hypertensive rats. *Am J Physiol Heart Circ Physiol* 2005;288:H2289-97
45. Schupp M, Janke J, Clasen R, et al. Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. *Circulation* 2004;109:2054-7
46. Skeggs LT Jr, Kahn JR, Lentz K, Shumway NP. The preparation, purification, and amino acid sequence of a polypeptide renin substrate. *J Exp Med* 1957;106:439-53
47. Jensen C, Herold P, Brunner HR. Aliskiren: the first renin inhibitor for clinical treatment. *Nat Rev Drug Discov* 2008;7:399-410
48. Lee HY, Oh BH. Cardio-renal protection with aliskiren, a direct renin inhibitor, in the ASPIRE HIGHER program. *Expert Rev Cardiovasc Ther* 2009;7:251-7
49. Rocha R, Funder JW. The pathophysiology of aldosterone in the cardiovascular system. *Ann N Y Acad Sci* 2002;970:89-100
50. Funder JW. Aldosterone and mineralocorticoid receptors in the cardiovascular system. *Prog Cardiovasc Dis* 2010;52:393-400
51. Fitzgerald N, Fitzgerald JD. Aldosterone antagonists: a model of translational medicine. *Dialogues Cardiovasc Med* 2009;14:118-25
52. Jeunemaitre X, Chatellier G, Kreft-Jais C, et al. Efficacy and tolerance of spironolactone in essential hypertension. *Am J Cardiol* 1987;60:820-5
53. MacFadyen RJ, Lee AF, Morton JJ, et al. How often are angiotensin II and aldosterone concentrations raised during chronic ACE inhibitor treatment in cardiac failure? *Heart* 1999;82:57-61
54. Pitt B, Zannad F, Remme WJ, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* 1999;341:709-17
- **RALES study showing surprisingly beneficial effects of spironolactone.**
55. Pitt B, White H, Nicolau J, et al. Eplerenone reduces mortality 30 days after randomization following acute myocardial infarction in patients with left ventricular systolic dysfunction and heart failure. *J Am Coll Cardiol* 2005;46:425-31
- **EPHESUS study showing beneficial effects of eplerenone.**
56. Williams GH, Burgess E, Kolloch RE, et al. Efficacy of eplerenone versus enalapril as monotherapy in systemic hypertension. *Am J Cardiol* 2004;93:990-6
57. Weinberger MH, White WB, Ruilope LM, et al. Effects of eplerenone versus losartan in patients with low-renin hypertension. *Am Heart J* 2005;150:426-33
58. White WB, Duprez D, St HR, et al. Effects of the selective aldosterone blocker eplerenone versus the calcium antagonist amlodipine in systolic hypertension. *Hypertension* 2003;41:1021-6
59. Krum H, Nolly H, Workman D, et al. Efficacy of eplerenone added to renin-angiotensin blockade in hypertensive patients. *Hypertension* 2002;40:117-23
60. Struthers A, Krum H, Williams GH. A comparison of the aldosterone-blocking agents eplerenone and spironolactone. *Clin Cardiol* 2008;31:153-8
61. Muldowney JA III, Schoenhard JA, Benge CD. The clinical pharmacology of eplerenone. *Expert Opin Drug Metab Toxicol* 2009;5:425-32
62. Yamaji M, Tsutamoto T, Kawahara C, et al. Effect of eplerenone versus spironolactone on cortisol and

- hemoglobin A(c) levels in patients with chronic heart failure. *Am Heart J* 2010;160:915-21
63. Weinberger MH, Roniker B, Krause SL, Weiss RJ. Eplerenone, a selective aldosterone blocker, in mild-to-moderate hypertension. *Am J Hypertens* 2002;15:709-16
64. Violin JD, DeWire SM, Yamashita D, et al. Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* 2010;335:572-9
- **First description of the *in-vivo* effects of biased agonism for the AT1 receptor.**
65. Rakesh K, Yoo B, Kim IM, et al. beta-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress. *Sci Signal* 2010;3:ra46
66. Duchene J, Bader M. Bradykinin B2 receptor agonism: A novel therapeutic strategy for myocardial infarction? *Am J Hypertens* 2010;23:459
67. Kuwahara K, Nakao K. Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones. *Endocr J* 2010;57:555-65
68. Campbell DJ. Vasopeptidase inhibition: a double-edged sword? *Hypertension* 2003;41:383-9
- **Comprehensive discussion of problems with vasopeptidase inhibition.**
69. Gros C, Noel N, Souque A, et al. Mixed inhibitors of angiotensin-converting enzyme (EC 3.4.15.1) and enkephalinase (EC 3.4.24.11): rational design, properties, and potential cardiovascular applications of glycopril and alatriopril. *Proc Natl Acad Sci USA* 1991;88:4210-14
70. Jullien N, Makritis A, Georgiadis D, et al. Phosphinic tripeptides as dual angiotensin-converting enzyme C-domain and endothelin-converting enzyme-1 inhibitors. *J Med Chem* 2010;53:208-20
71. Thorin E, Clozel M. The cardiovascular physiology and pharmacology of endothelin-1. *Adv Pharmacol* 2010;60:1-26
72. Akif M, Schwager SL, Anthony CS, et al. Novel mechanism of inhibition of human angiotensin-I-converting enzyme (ACE) by a highly specific phosphinic tripeptide. *Biochem J* 2011;436:53-9
73. Gu J, Noe A, Chandra P, et al. Pharmacokinetics and pharmacodynamics of LCZ696, a novel dual-acting angiotensin receptor-neprilysin inhibitor (ARNi). *J Clin Pharmacol* 2010;50:401-14
74. Lucchese B. Hypertension: LCZ696, a novel dual inhibitor of neprilysin and the angiotensin II receptor, shows promise in phase II trial. *Nat Rev Nephrol* 2010;6:383
75. Iwata N, Tsubuki S, Takaki Y, et al. Metabolic regulation of brain Abeta by neprilysin. *Science* 2001;292:1550-2
76. Becker M, Siems WE, Kluge R, et al. New function for an old enzyme: NEP deficient mice develop late-onset obesity. *PLoS One* 2010;5(9):e12793. doi:10.1371/journal.pone.0012793
77. Martelli A, Breschi MC, Calderone V. Pharmacodynamic hybrids coupling established cardiovascular mechanisms of action with additional nitric oxide releasing properties. *Curr Pharm Des* 2009;15:614-36
78. Nguyen G. Renin, (pro)renin and receptor: an update. *Clin Sci (Lond)* 2011;120:169-78
- **Most recent update on (pro)renin receptor research.**
79. Scheife JH, Menk M, Reinemund J, et al. A novel signal transduction cascade involving direct physical interaction of the renin/prorenin receptor with the transcription factor promyelocytic zinc finger protein. *Circ Res* 2006;99:1355-66
80. Inagami T, Nakagawa T, Ichihara A, et al. Renin/prorenin receptor, (P)RR, in end-organ damage: current issues in 2007. *J Am Soc Hypertens* 2008;2:205-9
81. Ichihara A, Hayashi M, Kaneshiro Y, et al. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. *J Clin Invest* 2004;114:1128-35
82. Funke-Kaiser H, Zollmann FS, Scheife JH, Unger T. Signal transduction of the (pro)renin receptor as a novel therapeutic target for preventing end-organ damage. *Hypertens Res* 2010;33:98-104
83. Nariai T, Fujita K, Mori M, et al. SM-368229, a novel selective and potent non-steroidal mineralocorticoid receptor antagonist with strong urinary Na(+) excretion activity. *J Pharmacol Sci* 2011;115:346-53
84. Paulis L, Unger T. Novel therapeutic targets for hypertension. *Nat Rev Cardiol* 2010;7:431-41
85. Fiebeler A, Nussberger J, Shagdarsuren E, et al. Aldosterone synthase inhibitor ameliorates angiotensin II-induced organ damage. *Circulation* 2005;111:3087-94
86. Lea WB, Kwak ES, Luther JM, et al. Aldosterone antagonism or synthase inhibition reduces end-organ damage induced by treatment with angiotensin and high salt. *Kidney Int* 2009;75:936-44
87. Mulder P, Mellin V, Favre J, et al. Aldosterone synthase inhibition improves cardiovascular function and structure in rats with heart failure: a comparison with spironolactone. *Eur Heart J* 2008;29:2171-9
88. Amar L, Azizi M, Menard J, et al. Aldosterone synthase inhibition with LCI699: a proof-of-concept study in patients with primary aldosteronism. *Hypertension* 2010;56:831-8
89. Funder JW. Mineralocorticoid receptors: distribution and activation. *Heart Fail Rev* 2005;10:15-22
90. Brown MJ. Success and failure of vaccines against renin-angiotensin system components. *Nat Rev Cardiol* 2009;6:639-47
- **Comprehensive review about vaccines against angiotensins.**
91. Tissot AC, Maurer P, Nussberger J, et al. Effect of immunisation against angiotensin II with CYT006-AnGQb on ambulatory blood pressure: a double-blind, randomised, placebo-controlled phase IIa study. *Lancet* 2008;371:821-7
- **First description of vaccination against angiotensin.**
92. Kuba K, Imai Y, Rao S, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* 2005;11:875-9
93. Imai Y, Kuba K, Rao S, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature* 2005;436:112-16
94. Ferreira AJ, Shenoy V, Qi Y, et al. Angiotensin-converting enzyme 2 activation protects against hypertension-induced cardiac fibrosis

Emerging drugs which target the renin–angiotensin–aldosterone system

- involving extracellular signal-regulated kinases. *Exp Physiol* 2011;96:287-94
95. Lula I, Denadai AL, Resende JM, et al. Study of angiotensin-(1-7) vasoactive peptide and its beta-cyclodextrin inclusion complexes: complete sequence-specific NMR assignments and structural studies. *Peptides* 2007;28:2199-210
96. Fraga-Silva RA, Costa-Fraga FP, Sousa FB, et al. An orally-active formulation of angiotensin-(1-7) produces antithrombotic effect. *Clinics* 2011;In press
97. Savergnini SQ, Beiman M, Lautner RQ, et al. Vascular relaxation, antihypertensive effect, and cardioprotection of a novel peptide agonist of the Mas receptor. *Hypertension* 2010;56:112-20
98. Marques FD, Ferreira AJ, Sinisterra RD, et al. An oral formulation of angiotensin-(1-7) produces cardioprotective effects in infarcted and isoproterenol-treated rats. *Hypertension* 2011;57:477-83
99. Wiemer G, Dobrucki LW, Louka FR, et al. AVE 0991, a nonpeptide mimic of the effects of angiotensin-(1-7) on the endothelium. *Hypertension* 2002;40:847-52
• **First description of a synthetic Mas agonist.**
100. Wan Y, Wallinder C, Plouffe B, et al. Design, synthesis, and biological evaluation of the first selective nonpeptide AT2 receptor agonist. *J Med Chem* 2004;47:5995-6008
• **First description of a non-peptide AT2 agonist.**
101. Steckelings UM, Larhed M, Hallberg A, et al. Non-peptide AT2-receptor agonists. *Curr Opin Pharmacol* 2011;11:187-92
102. Esler MD, Krum H, Sobotka PA, et al. Renal sympathetic denervation in patients with treatment-resistant hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. *Lancet* 2010;376:1903-9
• **First study about renal nerve ablation.**
103. Radonic T, De Witte P, Baars MJ, et al. COMPARE study group: Losartan therapy in adults with Marfan syndrome: study protocol of the multi-center randomized controlled COMPARE trial. *Trials* 2010;12:11-3104
104. Habashi JP, Doyle JJ, Holm TM, et al. Angiotensin II type 2 receptor signaling attenuates aortic aneurysm in mice through ERK antagonism. *Science* 2011;332:361-5

Affiliation

Ulrike Muscha Steckelings¹, Ludovit Paulis^{1,2}, Thomas Unger¹ & Michael Bader^{†3}

[†]Author for correspondence

¹Charité - University Medicine Berlin, Center for Cardiovascular Research, Berlin, Germany

²Comenius University, Institute of Pathophysiology, Faculty of Medicine, Bratislava, Slovakia Republic

³Max-Delbrück-Center for Molecular Medicine (MDC), Berlin-Buch, 13092 Berlin, Germany
Tel: +49 30 9406 2193; Fax: +49 30 9406 2110;
E-mail: mbader@mdc-berlin.de