Angiotensin converting enzyme-2 (ACE2) and its possible roles in hypertension, diabetes and cardiac function

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Summary

Angiotensin converting enzyme-2 (ACE2) is a recently described homologue of the vasoactive peptidase, angiotensin converting enzyme (ACE). Like ACE, ACE2 is an integral (type I) membrane zinc metallopeptidase, which exists as an ectoenzyme. ACE2 is less widely distributed than ACE in the body, being expressed at highest concentrations in the heart, kidney and testis. ACE2 also differs from ACE in its substrate specificity, functioning exclusively as a carboxypeptidase rather than a peptidyl dipeptidase. A key role for ACE2 appears to be emerging in the conversion of angiotensin II to angiotensin (1–7), allowing it to act as a counter-balance to the actions of ACE. ACE2 has been localised to the endothelial and epithelial cells of the heart and kidney where it may have a role at the cell surface in hydrolysing bioactive peptides such as angiotensin II present in the circulation. A role for ACE2 in the metabolism of other biologically active peptides also needs to be considered. ACE2 also serendipitously appears to act as a receptor for the severe acute respiratory syndrome (SARS) coronavirus. Studies using *ace2^{-/-}* mice, and other emerging studies *in vivo* and *in vitro*, have revealed that ACE2 has important functions in cardiac regulation and diabetes. Together with its role as a SARS receptor, ACE2 is therefore likely to be an important therapeutic target in a diverse range of disease states.

Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2; Ang, angiotensin; APJ, apelin receptor; AT1, AT2, AT4 (angiotensin receptor type 1,2,4); BK, bradykinin; HEK, Human embryonic kidney; HIV, human immunodeficiency virus; QTL, quantitative trait locus; RAS, renin-angiotensin system; SARS, severe acute respiratory syndrome; SHR, spontaneously hypertensive rat; SHRSP, spontaneously hypertensive rat (stroke-prone).

Introduction

Hypertension and diabetes are among leading causes of end-stage renal disease in most regions of the Western world. Moreover, they contribute to the progression of cardiovascular disease [1, 2]. Angiotensin I-converting-enzyme (ACE) inhibitors have been shown to have the broadest impact of any drug in cardiovascular medicine, reducing the risk of death, stroke, myocardial infarction, diabetes, and renal disease [2, 3]. ACE inhibition results in direct cardiovascular protective effects by lowering levels of angiotensin II (Ang II) and through increasing bradykinin availability. Constant reduction of blood pressure is considered the most beneficial effect of ACE inhibitors in both experimental and clinical studies of progressive diabetic

and non-diabetic renal disease [4, 5]. In addition to important haemodynamic effects such as reduced systemic blood pressure and glomerular capillary pressure, inhibition of the renin-angiotensin system (RAS) also produces important non-haemodynamic benefits such as stimulation of extracellular matrix degradation and blockade of macrophage/monocyte permeation of glomerular vascular beds [1]. Hence, efforts have been focused on understanding the physiological role of ACE, design of ACE inhibitors, and its interaction with other components of the RAS.

Angiotensin II and the renin-angiotensin system

Angiotensin II (Ang II), the principal effector of the RAS, causes vasoconstriction both directly and indirectly by stimulating angiotensin type 1 (AT1) receptors present on the vasculature and by increasing sympathetic tone and stimulating arginine vasopressin release. Long-term, blood pressure is regulated by Ang II by modulating sodium and water reabsorption through direct stimulation of AT1 receptors in the kidney, or indirectly through the production and release of aldosterone from the adrenal glands or thirst via a central action [6, for review]. The classical enzymatic pathway by which Ang II is formed begins with the cleavage of precursor angiotensinogen by renin, an aspartic proteinase, to the decapeptide angiotensin I (Ang I). The cascade continues with ACE cleaving a dipeptide from the C-terminus of Ang I to form Ang II. The actions of Ang II are mediated via specific G-protein-coupled receptors. In humans, two angiotensin receptors have been described, AT1 and AT2, that bind Ang II with differing affinities [6]. Angiotensin II is further cleaved by a variety of enzymes to produce the bioactive angiotensin fragments, angiotensin III (Ang 2-8), angiotensin IV (Ang 3-8) and angiotensin (1-7) {Ang (1-7)} [7]. Whilst this depiction (Figure 1) represents the RAS described in textbooks, the role of these other angiotensin fragments, the enzymes (ACE2, neprilysin (NEP), aminopeptidase A, prolyl carboxypeptidase and prolyl endopeptidase) implicated in their formation and the identification of new angiotensin receptors (AT4 and Mas) have all been pivotal in redefining the RAS in the last decade. Of particular interest to this review is ACE2, and its possible physiological roles in the control of blood pressure, renal disease and cardiac function.
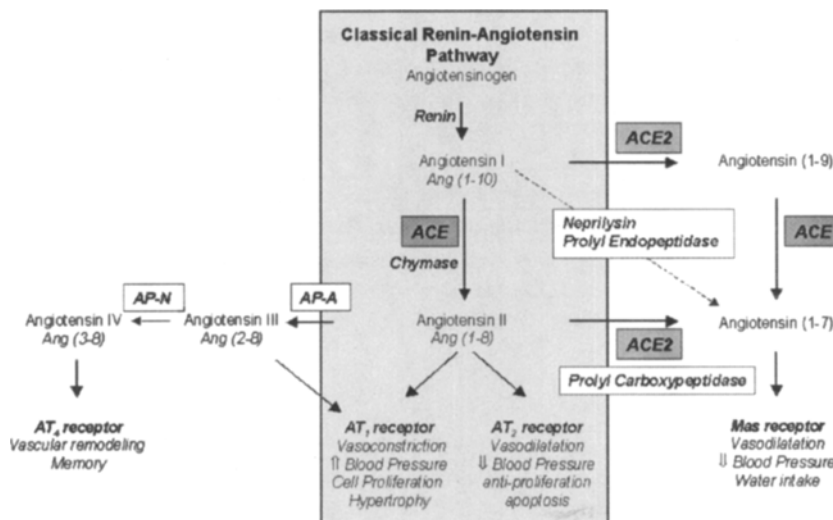
Figure 1. Renin-angiotensin System. The last 10 years has seen the discovery of new components that interface with the classical depiction of the renin-angiotensin system and formation of its physiological end-product, Angiotensin II (encased in grey box). Angiotensin II is metabolised to several bioactive fragments, Ang (1–7), Ang III and Ang IV by different enzymatic pathways. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AP-A aminopeptidase A; AP-N, aminopeptidase N.

Angiotensin converting enzyme-2

ACE2 is the first human homologue of ACE to be described [8, 9] and is also now known to serve as a functional receptor for the Severe Acute Respiratory Syndrome (SARS) coronavirus [10]. To date, relatively little is known of the cell biology of ACE2 or regulation of its expression in normal and pathological conditions. Northern blot analysis revealed highest levels of ACE2 expression in kidney, heart and testis [8, 9]. More extensive surveys have shown detectable levels of ACE2 expression in other tissues, especially the gastrointestinal tract [11]. However, in human primary and transformed vascular and kidney cell lines examined to date, no abundant source of ACE2 protein expression has emerged with ACE2 levels generally being an order of magnitude lower than those of ACE (F. J. Warner and A. J. Turner, unpublished observations). When expressed stably in Human Embryonic Kidney cells, ACE2 is found to be expressed predominantly at the cell-surface as an ectoenzyme, consistent with its existence, like ACE, as a type I integral membrane peptidase (Figure 2). Its cell-surface location is also consistent with its ability to be shed from the membrane as a soluble protein [9, D.W. Lambert, N.M. Hooper and A.J. Turner, unpublished observations], as is ACE [12], and to serve as a viral receptor [10].

Substrate specificity

ACE2 has been classified as a member of the M2 zinc metalloproteinase family along with somatic and testicular forms of ACE. The 805 amino acid sequence of ACE2 displays approximately 42% identity to human somatic ACE in the regions surrounding the catalytic active sites [8]. Unlike ACE, however, ACE2 contains only a single catalytic domain compared with the two active sites (N- and C-domains) of somatic ACE. Furthermore, ACE2 is a carboxypeptidase rather than a peptidyl dipeptidase, being the first mammalian carboxypeptidase to be identified that contains the HEXXH motif rather than the typical carboxypeptidase A-like motif, $HXXE(X)_{123-132}H$. ACE2 cleaves a single hydrophobic or basic residue from the C-terminus of a limited range of biologically active peptides [13, 14]. Indeed, of
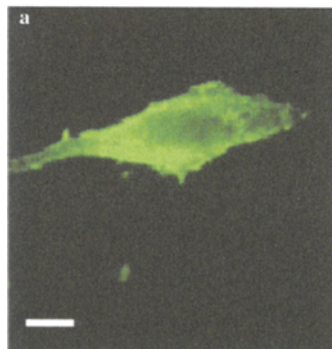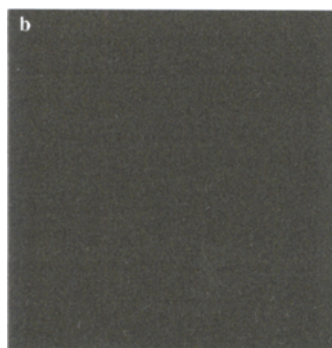
Figure 2. Immuofluorescent staining of (a, b) Human Embryonic Kidney cells transiently expressing full-length ACE2. Cells grown on coverslips were fixed using 4% w/v paraformaldehyde prior to immunostaining with polyclonal anti-peptide antibodies directed to sequences in the (a) extracellular (C-62 N-terminal antibody) (1:100) or (b) cytoplasmic (C-65 C-terminal antibody (1:100)) regions of ACE2. Donkey anti-rabbit FITC conjugate (1:500) was used as secondary. Untransfected HEK cells and transfected cells stained in the absence of primary antibody displayed negative immunostaining for ACE2 (not shown). 63× Bar, 2 μm.

126 peptides tested, only 11 served as ACE2 substrates *in vitro* under the assay conditions used [13]. As a consequence, the substrate specificity of ACE2, and hence its physiological roles, differ from those of ACE [15], nor is ACE2 inhibited by classical ACE inhibitors such as captopril, lisinopril or enalaprilat, *in vitro* [8, 16]. Two strategies have been used to develop selective inhibitors of ACE2. Dales *et al.* [17] employed a substrate-based design, whereas Huang *et al.* [18] used peptide library selection using phage display technology. Both strategies led to the identification of inhibitors of nanomolar potency against ACE2 that did not affect ACE activity.

The recent solution of crystal structures for human ACE2 ectodomain [19] and testicular ACE (tACE) [20] as well as comparative modelling of the ACE2 active site [14] indicate the catalytic mechanism of ACE2 closely resembles that of tACE. However, differences in substrate specificity and inhibitor sensitivity suggest distinctive differences exist between the substrate binding subsites of ACE2 and tACE. ACE2 cleaves a single amino acid from the C-terminus of its substrates, whereas ACE functions as a peptidyl dipeptidase. The basis for this difference relates primarily to the specificity pocket (S2' subsite) and binding of the substrate C-terminus. In ACE2, the S2' subsite is smaller as a result of the R273 $\Rightarrow$ Q substitution and is only able to accommodate one amino acid instead of a second terminal peptide bond, as compared with that of tACE [14, 19]. The action of ACE inhibitors such as captopril and lisinopril, is based, in part, upon their ability to mimic the dipeptidyl C-terminal binding of ACE substrates [3].

Amongst the substrates cleaved efficiently by ACE2 are angiotensin II, dynorphin A(1–13) and the apelin peptides, apelin-13 and apelin-36. Angiotensin I, des-Arg^9-bradykinin, β-casomorphin, and ghrelin are also hydrolysed by ACE2, although to a lesser extent [13, 14]. The N-domain specific ACE substrate and haemoregulatory peptide, N-Acetyl-Ser-Asp-Lys-Pro [21], and the vasodilator bradykinin are not cleaved by ACE2, although (des-Arg^9)-bradykinin is an ACE2 substrate in vitro [13]. Hence, it is possible that ACE2 may terminate the in vivo actions of

(des-Arg^9)-bradykinin, which is the endogenous ligand of the bradykinin B$_1$ receptor [more detail 15]. Some of the differences in substrate specificity between ACE and ACE2 are summarised in Table 1. Pertaining to the RAS, two angiotensin fragments produced through ACE2 cleavage of either Ang II or Ang I are Ang (1–7) and Ang (1–9), respectively. Of these two pathways, the conversion of Ang II to Ang (1–7) by ACE2 is kinetically substantially more favourable both in vitro [13, 14, 16, 22] and in vivo in humans [23]. Studies in vitro show ACE2 to be 10- to 600-fold more potent in hydrolysing Ang II to Ang (1–7) than prolyl endopeptidase and prolyl carboxypeptidase [24]. It is hence this ability of ACE2 to form Ang (1–7), a vasodilator, that has attracted most attention. There is increasing evidence to suggest that the majority of effects produced by the heptapeptide, Ang (1–7) are in counteracting the pressor, proliferative and profibrotic actions of Ang II and thereby providing a mechanism for Ang II counter-regulation [25]. These effects include vasodilatation, anti-proliferation [26, 27], increased baroreflex sensitivity [28], potentiation of bradykinin activity at B2 receptors [25, 29, 30, 31, for review] and inhibition of C-domain ACE activity [32]. It should also be noted that in addition to the Ang II counter-regulatory effects of Ang (1–7), this peptide has been shown to produce effects that are either similar [33, 34, 35, 36] or distinct from those of Ang II [37, 38].

Santos and co-workers [39] have recently demonstrated that Ang (1–7) is the endogenous ligand

Table 1. A comparison of the hydrolysis of some peptide substrates by ACE and ACE2

Substrate	ACE	ACE2
Angiotensin I	DRVYIHPF $\downarrow$ HL	DRVYIHPFH $\downarrow$ L
Angiotensin II	not cleaved	DRVYIHP $\downarrow$ F
Angiotensin (1–9)	DRVYIHP $\downarrow$ FH	not cleaved
Angiotensin (1–7)	DRVYI $\downarrow$ HP	not cleaved
Bradykinin	RPPGFSP $\downarrow$ FR	not cleaved
des-Arg^9 Bradykinin	not cleaved	RPPGFSP $\downarrow$ F
Dynorphin A (1–13)	not cleaved	YGGFLRRIRPKL $\downarrow$ K
Apelin-13	not determined	QRPRLSHKGPMP $\downarrow$ F
Ghrelin	not determined	.... ESKKPPAKLQP $\downarrow$ R
AcSKDP	Ac-SK $\downarrow$ DP	not cleaved

The sequence of each peptide is indicated using single-letter coding for amino acids. An arrow indicates the peptide bond hydrolysed. Data cited from [8, 9, 13, 14].

for the G-protein coupled receptor, Mas. Extensive studies have been performed to understand which receptors (AT1, AT2, non-AT1/AT2) and/or other mechanisms (ACE inhibition and ACE-bradykinin B2 receptor 'crosstalk') are involved in mediating the effects of Ang (1–7) [25, for review]. The identification of a functional receptor for Ang (1–7) has clinical implications. In humans and rats, administration of ACE inhibitors leads to an accumulation of Ang (1–7); ACE blockade prevents degradation of Ang (1–7) to Ang (1–5), whilst Ang (1–7) continues to be generated by non-ACE pathways (Figure 1) [40, 41]. Future investigations are now required to decipher which of the known Ang (1–7) effects can be attributed to interaction with the Mas receptor.

The relationship between ACE2 and blood pressure

Within the RAS, it is hypothesised that ACE2 generates Ang (1–7) through hydrolysis of Ang II, thereby maintaining a state of normal tension by limiting the vasopressor effects of Ang II as well as its hypertrophic and pro-oxidative effects. It should also be noted that the bioavailability of Ang I is also a regulatory step in the formation of Ang II. In normal subjects, an inverse correlation between Ang (1–7) and arterial pressure has been reported [42]. Several authors have speculated that ACE2 may be an important regulator of blood pressure homeostasis and contribute to the pathophysiology of essential hypertension [43, 44]. This suggestion is based upon genetic studies [43, 45], as well as data obtained both in vitro and in vivo [18, 43, 46]. In humans, the ace2 gene is located in a region of the X chromosome (Xp22) [8]. On the rat X chromosome, the ace2 gene maps to a defined quantitative trait locus (QTL) that is associated with hypertension and has been previously identified in rat models of spontaneous or diet-induced hypertension [43]. This observation suggests ace2 may be a candidate gene for this hypertension-related QTL. Furthermore, single nucleotide polymorphisms have been identified in the ACE2 locus and were associated with a number of cardiovascular endpoints: percutaneous transliminal coronary angioplasty, coronary artery bypass grafting, myocardial infarction and catheterisation in patients with > 70% stenosis [45], but not essential hypertension [62].

In rat models of hypertension, the levels of kidney ACE2 mRNA and protein expression is markedly lower than that observed in normotensive controls [43]. Based upon the hypothesis mentioned above, reduced levels of ACE2 increase Ang II availability and reduce Ang (1–7) formation, and therefore lead to an increase in blood pressure. This trend is observed in vitro in hypertensive rat models. The baseline systolic blood pressure of salt-sensitive Sabra hypertensive rats (SBH/y) is 10–15 mm Hg higher compared to that of the control salt-resistant Sabra normotensive rats (SBN/y). Upon salt-loading a further decrease in ACE2 expression in conjunction with an increase in systolic blood pressure is observed, whilst no change is noted in control animals. A further example of this trend is also reported in spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rat strains (SHRSP), which present hypertension without any obvious external stimulus. SHR and SHRSP consistently have reduced kidney ACE2 expression compared to those levels seen in Wistar-Kyoto control rats [43].

Whilst both genetic and in vitro data follow this apparent trend quite closely, the data produced from in vivo ace2 knockout mice are less obvious. In ace2$^{-/-}$ mice, Allred and co-workers [46] reported higher systolic blood pressures (5 mm Hg) in ACE2 null mice than in control littermates. This increase in blood pressure is further exacerbated by chronic infusion with Ang II. This reported increase in blood pressure, although only preliminary data published in an abstract form [46], is consistent with findings in the Sabra model in which SBH/y rats with lower ACE2 expression have a higher baseline blood pressure [43]. Furthermore, Allred et al. [46] also observed three-fold higher Ang II levels in the serum of ace2 null mice compared to control mice following Ang II infusion. This observation is in accord with the current hypothesis that ACE2 regulates the level of Ang II in vivo and maintains blood pressure homeostasis. In contrast, no change in blood pressure was observed in 3-month old ace2 null mice compared with control littermates, despite there being an increase in kidney, heart and plasma Ang II

levels [43]. At 6 months old, *ace2* null mice of both genders displayed impaired heart function, which in male mice coincided with a slight reduction in blood pressure. This result highlights a gender-specific effect of X-linked ACE2 on the cardiovascular system, but secondly suggests other genetic factors may also contribute to the effects of ACE2. The aetiology of essential hypertension is considered multi-factorial being influenced by genetic as well as environmental factors. The somewhat contradictory results of these two knockout mouse studies by Allred *et al.* [46] and Crackower *et al.* [43] may originate from differences in the genetic make-up, which greatly influences the phenotype of gene knockouts.

To add further confusion to the above hypothesis, the blocking of ACE2 activity via intravenous bolus injection (3 mg/kg) of the selective ACE2 inhibitor, DX512 (K_i, 139 nM) in conscious SHR produces a dose-dependent depressor response [18]. This response was characterised by a decreased mean arterial pressure (maximal average depressor response 70.5 mm Hg from an average mean arterial pressure 155 mm Hg) and reflex tachycardia [18]. These experiments warrant further investigation since they disagree with both the *in vitro* studies in SHR rats [43] and *in vivo* data discussed above showing either an increase or no change in systolic blood pressure [43, 46]. It may be possible that this inhibitor is potentiating the effects of a substrate other than Ang II. For instance, apelin-13 is hydrolysed by ACE2 with comparable kinetics to Ang II [13] and has been shown to produce hypotensive effects and increase water intake [47, 48], through interaction with its endogenous receptor, APJ (a putative receptor related to AT1 and a co-receptor for the HIV-1 virus) [49]. Overall, this contradiction of results *in vivo* between *ace2* gene ablation and the effect of an ACE2 inhibitor provides an excellent example of fundamental differences that exist between knockout animals, which have never produced ACE2 and may have developed compensatory mechanisms, and animals in which ACE2 is inhibited for only a short span of time.

ACE2 and its role in the kidney and diabetes

In the kidney, ACE2 displays a similar localisation pattern to that observed for ACE being primarily localised to both distal and proximal renal tubules and rarely to glomerular cells in rats and humans [9, 50, 51, 52]. Whilst it is hypothesised that ACE2 may regulate blood pressure through conversion of Ang II to Ang (1–7), it is unclear if ACE2 has an important role in renal function and homeostasis. Two studies have emerged investigating a role for ACE2: the first in diabetes [51] and the second in the production of Ang (1–7) during pregnancy [52]. In the animal models studied by Tikellis *et al.* [51] and Brosnihan *et al.* [52], there was an absence of hypertension, suggesting ACE2 may have a role in the local production of Ang (1–7) and in the intrarenal RAS within the kidney. Moreover, these studies provide the first evidence of the presence of ACE2 activity within the kidney. In a rat model of diabetic nephropathy, gene and protein expression of tubule ACE2 were observed to decrease in line with ACE [51]. However, for both ACE2 and ACE there was an increase in protein expression in diabetic glomeruli [51]. This finding is in keeping with the observation that ACE is redistributed towards glomeruli and renal vasculature and away from the proximal tubules in diabetic rat and human kidney [53, 54]. Furthermore, this may suggest a role for glomerular ACE, and possibly ACE2, in mediating nephron injury by increasing intraglomerular Ang II formation. Intrarenal expression of Ang II is increased in diabetic nephropathy despite suppression of the systemic RAS [55]. An increase in glomerular ACE may explain this increase in Ang II, despite there being a decrease in tubule ACE. The study by Brosnihan *et al.* [52] also supports the concept for an intrarenal RAS and presents data suggesting ACE2 has a prominent role in local production of Ang-(1–7) during pregnancy. Nevertheless, further study of ACE2 and its involvement in both systemic and local RAS systems is required.

The contribution of ACE2 to cardiac function, ventricular remodelling and heart failure

Whilst the majority of research has concentrated on a possible role for ACE2 in hypertension and counteracting the actions of ACE, a

new field of research has emerged identifying a role for ACE2 in cardiac function, especially ventricular remodelling and heart failure. The first evidence that ACE2 may have a role in cardiac function was demonstrated by Crackower et al. [43] where the genetic ablation of *ace2* in mice resulted in a severe heart contractility defect, increased levels of Ang II in the kidney, heart and plasma and an up-regulation of hypoxia–induced genes in the heart. These findings led to the suggestion that ACE2 is an essential regulator of heart function [43]. However, it remains unclear if the RAS is involved in mediating this heart dysfunction. Studies where the RAS has been either blocked by ACE inhibitors or angiotensin receptor antagonists suggest the involvement of the RAS in the regulation of heart function and cardiac hypertrophy [56]. Neither *ace, angiotensinogen* nor *AT1 receptor* null mice show any defects in heart development and function [57, for review]. However, when *ace* is also ablated alongside *ace2* in mice, the cardiac phenotype of this double knockout is restored to that of wild-type littermates. It seems likely that a product of ACE may be responsible for the cardiac impairment observed in *ace2* null mice [43]. However, current literature would tend to suggest Ang II is not responsible for this cardiac defect, since Ang II promotes myocardial fibrosis and cardiac hypertrophy [58]. Further study of the effects of Ang II or the role of other candidate substrates is required.

Both the RAS and Ang II are key factors in ventricular remodelling associated with heart failure [59, for review]. In contrast to the *ace2* knockout study by Crackower et al. [43], cardiomyocyte-specific overexpression of *ace2* in mouse heart results in conduction disturbances and lethal ventricular arrhythmias. These outcomes were in association with a down-regulation of connexins, suggesting that overexpression of *ace2* disrupts gap junction formation [60]. Whilst there does not appear to be a clear link between the RAS and the loss of cardiac function observed in *ace2* null mice, Ang II has been implicated in gap junction dysregulation and development of conduction disturbances, which are thought to lead to lethal ventricular remodelling in heart failure [61]. Investigations of ACE2 and its role in cardiac function and heart failure will no doubt provide greater insight into the roles of this novel enzyme.

Concluding remarks

Since the first descriptions of ACE2 in 2000 [8, 9], information has rapidly accumulated on its possible functions from a variety of biochemical, genetic and physiological studies, yet much remains to be learned. It is clear that ACE2 has important roles in heart function, and possibly in blood pressure regulation and diabetes. It has also been identified as a SARS receptor. Hence, the diversity of its biological roles is perhaps the most surprising discovery in relation to this novel enzyme. It is now important to provide a much fuller description of the expression sites of the enzyme and the regulation of this expression. The identification of physiologically relevant peptide substrates, other than angiotensin II, is critical to a greater understanding of ACE2 biology. Finally, the involvement of ACE2 in human pathologies needs to be critically evaluated and its potential as a therapeutic target exploited where possible.

Acknowledgements

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