Experimental Physiology

Angiotensin-converting enzyme 2 catalytic activity in human plasma is masked by an endogenous inhibitor

Rebecca A. Lew[1], Fiona J. Warner[1], Iresha Hanchapola[1], Michael A. Yarski[1], Jay Manohar[2], Louise M. Burrell[2] and A. Ian Smith[1]

[1]*Department of Biochemistry & Molecular Biology, Monash University, Clayton, Victoria, Australia*
[2]*Department of Medicine, University of Melbourne, Austin Health, Heidelberg, Victoria, Australia*

Angiotensin-converting enzyme 2 (ACE2) is thought to act in an opposing manner to its homologue, angiotensin-converting enzyme (ACE), by inactivating the vasoconstrictor peptide angiotensin II and generating the vasodilatory fragment, angiotensin(1–7). Both ACE and ACE2 are membrane-bound ectoenzymes and may circulate in plasma as a consequence of a proteolytic shedding event. In this study, we show that ACE2 circulates in human plasma, but its activity is suppressed by the presence of an endogenous inhibitor. Partial purification of this inhibitor indicated that the inhibitor is small, hydrophilic and cationic, but not a divalent metal cation. These observations led us to develop a method for removal of the inhibitor, thus allowing detection of plasma ACE2 levels using a sensitive quenched fluorescent substrate-based assay. Using this technique, ACE2 activity measured in plasma from healthy volunteers ($n = 18$) ranged from 1.31 to 8.69 pmol substrate cleaved min^{-1} ml^{-1} (mean $\pm$ S.E.M., 4.44 ± 0.56 pmol min^{-1} ml^{-1}). Future studies of patients with cardiovascular, renal and liver disease will determine whether plasma ACE2 is elevated in parallel with increased tissue levels observed in these conditions.

(Received 21 October 2007; accepted after revision 9 January 2008; first published online 25 January 2008)
Corresponding author A. I. Smith: Department of Biochemistry & Molecular Biology, PO Box 13D, Monash University, Clayton, Victoria 3800, Australia. Email: ian.smith@med.monash.edu.au

The generation of angiotensin II (Ang II) within the renin–angiotensin system (RAS) is recognized as a critical point in the regulation of cardiovascular function. The final step in the production of Ang II is catalysed by the membrane-bound ectoenzyme angiotensin converting enzyme (ACE), and inhibitors of ACE are the most widely prescribed therapy in the treatment of hypertension, heart failure and myocardial infarction. Several years ago, the homologous enzyme ACE2 was discovered (Donoghue *et al.* 2000; Tipnis *et al.* 2000). Evidence suggests that ACE2 may work in a counter-regulatory role to ACE, via the inactivation of Ang II, and the formation of the putative vasodilator, Ang(1–7) (see Ferrario *et al.* 2005 for review). Within the cardiovascular system, ACE2 expression is normally relatively low and restricted to endothelial cells of the coronary and renal circulation, and epithelial cells of the renal distal tubules (Donoghue *et al.* 2000; Hamming *et al.* 2004; Burrell *et al.* 2005). However, we and others have shown that ACE2 levels in these and other tissues increase markedly in a number of pathologies, including myocardial infarction (Ishiyama *et al.* 2004; Burrell *et al.* 2005), athlerosclerosis (Zulli *et al.* 2006), diabetes (Ye *et al.* 2004), renal disease (Lely *et al.* 2004) and liver cirrhosis (Paizis *et al.* 2005), suggesting a role for the enzyme in limiting the damage associated with RAS activation in these conditions.

It has long been recognized that in addition to its localization on endothelial cell membranes, ACE is also present in plasma (Alhenc-Gelas *et al.* 1983; Hooper 1991). This soluble ACE arises from proteolytic 'shedding' of the membrane-bound enzyme (Parkin *et al.* 2004). Angiotensin-converting enzyme 2 is also shed from cells in culture (Lambert *et al.* 2005) and has been reported to circulate in plasma in rats (Ocaranza *et al.* 2006; Herath *et al.* 2007), sheep (Shaltout *et al.* 2007) and humans (Rice *et al.* 2006), as well as in transgenic, but not wild-type, mice (Donoghue *et al.* 2003). Levels of circulating ACE2 are very low compared with ACE levels and, in humans, ACE2 activity could only be detected in 7.5% of a large cohort of subjects (Rice *et al.* 2006). Our own initial attempts to measure ACE2 activity directly in plasma from healthy individuals were unsuccessful;

 DOI: 10.1113/expphysiol.2007.040352

however, during the course of these studies, we made the observation that plasma itself potently inhibits the activity of purified recombinant ACE2, suggesting the presence of an endogenous inhibitor of ACE2. In this report, we describe both the preliminary characterization of this inhibitory substance and the quantification of plasma ACE2 activity following inhibitor depletion by a simple anion exchange step.

Methods

Ethical approval

Research was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and was approved by the Research Ethics Committee at Austin Health. All subjects provided written informed consent for their involvement.

Plasma sampling

Blood was taken from normal, non-fasted, Caucasian volunteers ($n = 18$; 12 female, 6 male; age range 23–53 years, mean ± S.E.M. = 35 ± 1 year) recruited from the University of Melbourne and Austin Health, Melbourne, Australia. No subject had a past history of cardiac disease, and no subject was on medication. After 10 min rest, 5 ml blood was collected on ice into tubes containing lithium heparin, spun within 30 min at −4°C for 10 min, and plasma collected. All 18 samples were taken on a single morning, and plasma was stored at −70°C until assayed in a single batch (approximately 2 weeks between sampling and assay).

Expression and purification of soluble recombinant human ACE2

An expression construct for secreted soluble ACE2 was generated by fusing the interleukin-3 signal sequence and a Flag tag to the N-terminus of the extracellular catalytic domain of ACE2 (amino acids 2–738) and a hexa-histidine tag on the C-terminus (described by Douglas *et al.* 2004). The cDNA was ligated into pcDNA3.1 (Invitrogen Australia, Mount Waverley, Victoria, Australia) for transient transfection into HEK 293-T cells by the calcium phosphate method (Jordan *et al.* 1996). Secreted ACE2 was purified from media by sequential Ni-NTA (Ni^{3+} charged nitrilotriacetic acid-linked resin; Invitrogen) and anti-Flag (Sigma-Aldrich, Castle Hill, NSW, Australia) chromatography, according to the manufacturers' instructions.

Assay for ACE2 activity

The catalytic activity of ACE2 was measured as described previously (Douglas *et al.* 2004; Burrell *et al.* 2005; Warner *et al.* 2005), using an ACE2 quenched fluorescent substrate [QFS, (7-methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-dinitrophenyl; Vickers *et al.* 2002]. Cleavage of the QFS was attributed to ACE2 by the use of the specific inhibitor MLN-4760 at 100 nmol l^{-1} (a generous gift of Dr Natalie Dales, Millenium Pharmaceuticals, Cambridge, MA, USA; Dales *et al.* 2002). The rate of substrate cleavage was expressed as picomoles of substrate cleaved per minute per millilitre of plasma.

Assay for ACE activity

Plasma ACE activity was measured using a modification of a previously published method (Santos *et al.* 1985). Briefly, 2 μl plasma was incubated at 37°C with the ACE substrate hippuryl-His-Leu (1 mmol l^{-1}) in a total volume of 50 μl buffer (0.4 mol l^{-1} sodium borate buffer, 0.3 mol l^{-1} NaCl, pH 8.3), in the presence or absence of the ACE-specific inhibitor ramipril (10 μmol l^{-1}), for 30 min. Following incubation, 120 μl 0.3 N NaOH and 10 μl *o*-phthaldialdehyde (20 mg ml^{-1} in methanol) were added. After 10 min at room temperature, 20 μl 3 N HCl was added, the tubes were centrifuged at 16 000 **g** in a tabletop microcentrifuge for 5 min, and the supernatants transferred to a round-bottomed black 96-well microtitre plate. Fluorescence (excitation wavelength = 355 nm, emission wavelength = 485 nm) was measured using a FLUOstar Optima plate reader (BMG Labtechnologies, Durham, NC, USA). The rate of substrate cleavage was determined by comparison with a standard curve of the product His-Leu.

Characterization of plasma ACE2 inhibitor

Acetonitrile precipitation. Acetonitrile (ACN) was added to plasma to final concentrations of 20–60%, the precipitate removed, and the supernatant (resuspended in aqueous buffer after drying) tested for inhibition of ACE2. Cleavage of the QFS was confirmed by reverse-phase HPLC using standard methods (Lew 2003). In addition, the inhibitory effect of the 40% ACN plasma preparation on ACE2 cleavage of Ang II was also determined by HPLC. The potential inhibition of other metallopeptidases (neprilysin, thimet oligopeptidase and endothelin-converting enzyme) by the 40% ACN plasma preparation was tested using specific fluorometric assays (Steer *et al.* 2002; Kuruppu *et al.* 2007).

Solid-phase extraction. Fractionation of plasma was performed using disposable solid-phase extraction columns [C18 reverse-phase (Sep-Pak, Waters, Mitford, MA, USA), size-exclusion (PD-10, GE Healthcare, Uppsala, Sweden; exclusion limit 5000 Da), anion exchange (HiTrap anion exchange Sepharose 4 FastFlow, GE Healthcare) and cation exchange (HiTrap SP Sepharose XL, GE Healthcare)], and fractions were tested for inhibition of ACE2.

Chelation of divalent cations. An aliquot of the 40% ACN plasma preparation was treated with prewashed Chelex-100 resin (sodium form, 50–100 mesh, Sigma-Aldrich) in 20 mmol l^{-1} Tris-HCl buffer, pH 6.5, overnight at room temperature prior to determination of inhibition of recombinant ACE2 activity in the QFS assay. Control samples (also incubated overnight at room temperature) included the 40% ACN fraction in the absence of Chelex, 10 μmol l^{-1} $CuSO_4$ (which, when diluted $\times 2$ in the QFS assay, inhibited ACE2 by $\sim$70%), and Chelex-treated $CuSO_4$.

Preparation of plasma samples for ACE2 activity assay by anion exchange

Plasma (0.25 ml) was diluted into low-ionic-strength buffer (20 mmol l^{-1} Tris-HCl, pH 6.5) and added to 200 μl ANX Sepharose 4 Fast-Flow resin (Amersham Biosciences, GE Healthcare, Uppsala, Sweden). Following binding and washing, proteins were eluted with high-salt buffer (20 mmol l^{-1} Tris-HCl, 1 mol l^{-1} NaCl, pH 6.5), and the resulting eluate was assayed for ACE2 catalytic activity.

Immunoprecipitation

Angiotensin-converting enzyme 2 was immunodepleted from anion exchange eluates from selected plasma samples by a standard Protein G immunoprecipitation method, using a commercial polyclonal antiserum raised in goats against the ectodomain of human ACE2 (catalogue no. AF933, R&D Systems, Minneapolis, MN, USA) or normal goat serum used as a control for non-specific precipitation of ACE2. Non-precipitated supernatants were tested for ACE2 activity in the QFS assay.

Western immunoblot analysis

Following anion exchange extraction, plasma proteins were concentrated using a 30 kDa molecular weight cut-off Nanosep centrifugal concentrator (Pall Sciences, East Hills, NY, USA) prior to separation by SDS-polyacrylamide gel electrophoresis. A portion of the sample was deglycosylated using peptide: N-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA, USA) for 4 h, according to the manufacturer's instructions. Western immunoblot analysis was performed using a polyclonal anti-ACE2 antiserum (R&D Systems, catalogue no. AF933, diluted 1:100), as described previously (Lew *et al.* 2006).

Results

Human plasma contains an inhibitor of ACE2 activity

Catalytic activity of ACE2 was undetectable in normal human plasma when tested directly in the QFS assay; the lack of activity was not the result of non-specific quenching or interference with the fluorescent product, since the uncleaved substrate was confirmed by HPLC analysis. Indeed, addition of plasma to recombinant soluble human ACE2 inhibited activity in a dose-dependent manner (Fig. 1); this was confirmed by HPLC analysis. For example, a 10-fold dilution of plasma (10 μl in 100 μl) reduced the rate of QFS cleavage by approximately half. Similar results were observed for the full-length, membrane-bound form. Inhibition of ACE2 activity was not confined to the fluorescent substrate, since Ang II cleavage was similarly reduced by plasma or by a 40% acetonitrile-soluble fraction of plasma (Fig. 2). The inhibitor was present in similar amounts in plasma samples from individual volunteers, since the inhibition of recombinant ACE2 by 15 μl plasma was 37.7 $\pm$ 1.3% (mean $\pm$ S.E.M., $n = 17$). Finally, in separate screening experiments, we found that inhibition of ACE2 by plasma was independent of the anticoagulant used to collect the blood (lithium heparin, sodium citrate or plain blood collection tubes).

Characterization of the plasma ACE2 inhibitor

The inhibitory component of plasma remained soluble in ACN at concentrations up to 40% (Table 1); higher concentrations resulted in significant loss of inhibitory activity. This result suggests that the inhibitor is unlikely to be a large protein; this conclusion is supported by the

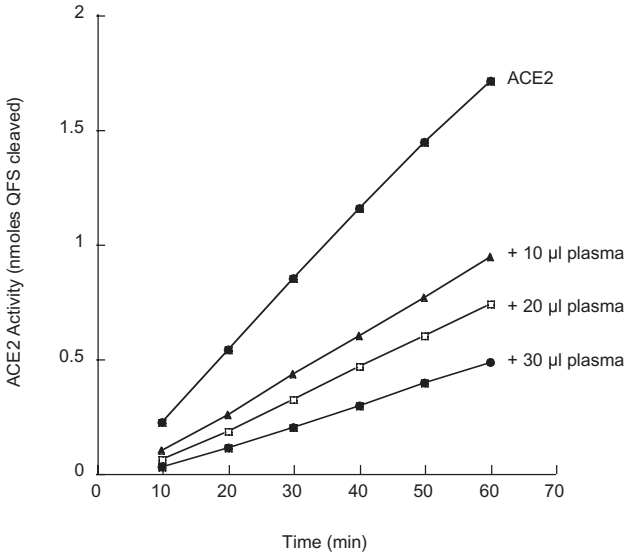

Figure 1. Inhibition of ACE2 activity by human plasma
Shown are the results of a representative experiment, in which plasma was added to soluble recombinant human ACE2 at the volumes indicated, in a final assay volume of 100 μl. Fluorescence resulting from the cleavage of an ACE2-specific quenched fluorescent substrate was continuously monitored, and the data expressed as nanomoles cleaved over time.

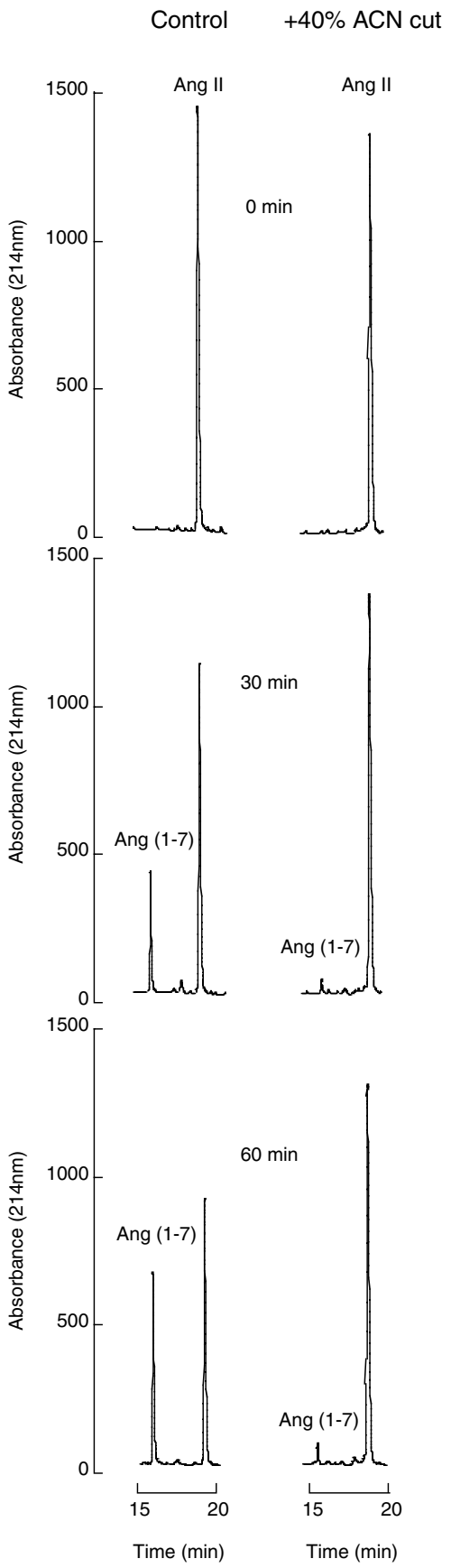

Table 1. Relative activity of recombinant ACE2 in the presence of plasma or acetonitrile (ACN) fractions of plasma

Sample	Relative activity (%)
Recombinant ACE2	100
+ Plasma	51.5
+ 20% ACN cut	56.7
+ 30% ACN cut	60.0
+ 40% ACN cut	54.9
+ 50% ACN cut	85.1
+ 60% ACN cut	93.9

The final dilution of plasma (or plasma equivalent) in the assay was 10-fold.

relative resistance of the reconstituted 40% ACN fraction to heating (80°C for 20 min), a treatment which resulted in the further precipitation of proteins but reduced the inhibition of ACE2 by only ∼30%. The 40% ACN fraction was also used to assess the specificity of the inhibition; whereas addition of this fraction (following drying and resuspension in aqueous buffer, equivalent to 50 μl neat plasma in a 100 μl total assay volume) to ACE2 inhibited activity by $> 80\%$, neprilysin and thimet oligopeptidase were unaffected, and endothelin-converting enzyme was inhibited by $< 25\%$. Fractionation of plasma by size exclusion chromatography (PD-10 column, exclusion limit 5000 Da) also indicates that the inhibitor is relatively small, since its elution was delayed (Table 2). Conversely, inhibitory activity was not retained on a C18 reverse-phase column (Sep-Pak; Table 2), suggesting a compound of low hydrophobicity. When applied to ion exchange columns, the inhibitor did not bind to the anion exchange medium and could not be eluted from the cation exchanger (Table 2), suggesting a cationic substance.

Preliminary experiments indicated that among the divalent cations found in plasma (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}), only Cu^{2+} (used as the sulphate salt) inhibited ACE2 with a relatively low IC_{50} of approximately 5 μmol l^{-1}. To determine whether the inhibitory substance in plasma was a divalent cation, we treated both the reconstituted 40% ACN preparation and a solution of 10 μmol l^{-1} $CuSO_4$ with the chelating resin Chelex-100. Whilst inhibition of ACE2 by a twofold dilution of the $CuSO_4$ (69% inhibition) was completely reversed by chelation, no change in the inhibitory effect of the plasma preparation was observed (52% inhibition), suggesting that the inhibitory substance is not a divalent cation.

Figure 2. Inhibition of ACE2 cleavage of Ang II to Ang(1–7) by a 40% acetonitrile-soluble fraction of plasma
Shown is the cleavage of Ang II to Ang(1–7) by recombinant ACE2 over time in the absence (Control) or presence of the 40% acetonitrile-soluble fraction of plasma (+40% ACN cut; 1:10 final dilution in digest).

Table 2. Elution of plasma inhibitory activity from solid-phase extraction columns

Column	Effect on ACE2
Reverse-phase (C18 Sep-pak)	
Fraction 1 (non-retained)	**Inhibition**
Fraction 2 (non-retained)	**Inhibition**
Fraction 3 (wash)	No inhibition
Fraction 4 (wash)	No inhibition
Fraction 5 (retained)	No inhibition
Fraction 6 (retained)	No inhibition
Size exclusion (PD-10)	
Fraction 1	No inhibition
Fraction 2	No inhibition
Fraction 3	No inhibition
Fraction 4	**Inhibition**
Fraction 5	**Inhibition**
Fraction 6	No inhibition
Anion exchange (ANX)	
Fraction 1 (non-retained)	**Inhibition**
Fraction 2 (wash)	No inhibition
Fraction 3 (high salt elution)	No inhibition
Cation exchange (SP)	
Fraction 1 (non-retained)	No inhibition
Fraction 2 (wash)	No inhibition
Fraction 3 (high salt elution)	No inhibition

Development of an anion exchange method to remove ACE2 inhibitor from plasma

Following the observation that the plasma inhibitor of ACE2 is not retained on anion exchange resin, we first verified that the recombinant enzyme itself does bind to this material and that recovery after elution is essentially 100% (data not shown). Pooled plasma from healthy volunteers was then used to determine whether endogenous ACE2 could be detected following anion exchange extraction and to assess the reproducibility of the assay. The inter- and intra-assay coefficients of variance were 13.7 ($n = 12$) and 7.1% ($n = 9$), respectively. Although we routinely measured ACE2 in 0.25 ml plasma samples, the method is sensitive enough to use with volumes as low as 0.1 ml.

In addition, aliquots of pooled plasma were heated to 60°C for 20 min to inactivate endogenous ACE2 and then spiked with varying concentrations of purified recombinant ACE2 (2–40 ng ml^{-1}) to create a standard curve for the semi-quantification of plasma ACE2 (Fig. 3; mean of 7 spiking experiments). Recovery of recombinant ACE2 activity was > 90%, with a strongly linear relationship between ACE2 added and measured activity (correlation coefficient, $r = 0.9996$).

The specificity of the assay method for ACE2 was verified by the use of the specific inhibitor MLN-4760, which inhibited the activity in anion exchange extracts of normal pooled plasma by 82.6 ± 2.3% ($n = 10$ separate extractions of pooled plasma) at a concentration (100 nmol l^{-1}) that completely blocks QFS cleavage by the recombinant enzyme. This result indicated that a small amount of the proteolytic activity against the QFS could not be attributed to ACE2; thus, the ACE2-specific activity was calculated by subtraction of the residual activity in the presence of the specific Millenium inhibitor MLN-4760. As an additional check for specificity, ACE2 was immunoprecipitated from anion exchange extracts of six representative plasma samples, which resulted in near-complete depletion of QFS-cleaving activity from the supernatant (Fig. 4).

Using Western immunoblot analysis, ACE2 was undetectable in straight plasma samples, even after concentration, in part owing to consistent smearing in the lane; however, following both anion exchange and concentration, a faint band was visible at ∼90 kDa, which was reduced to 83 kDa following PNGase F treatment (Fig. 5). An additional faint band was seen at ∼130 kDa, the same size as full-length membrane-bound recombinant protein; this band was not present in the PNGase F-treated sample (Fig. 5).

The activity of ACE2 in anion exchange-extracted plasma was measured in 18 samples from healthy volunteers and ranged from 1.31–8.69 pmol substrate cleaved min^{-1} ml^{-1} (Fig. 6; mean ± S.E.M. = 4.44 ± 0.56 pmol substrate cleaved min^{-1} ml^{-1}). These activity levels were approximately equal to those seen when heat-inactivated plasma was spiked with 10–40 ng recombinant ACE2 per millilitre. The activity in

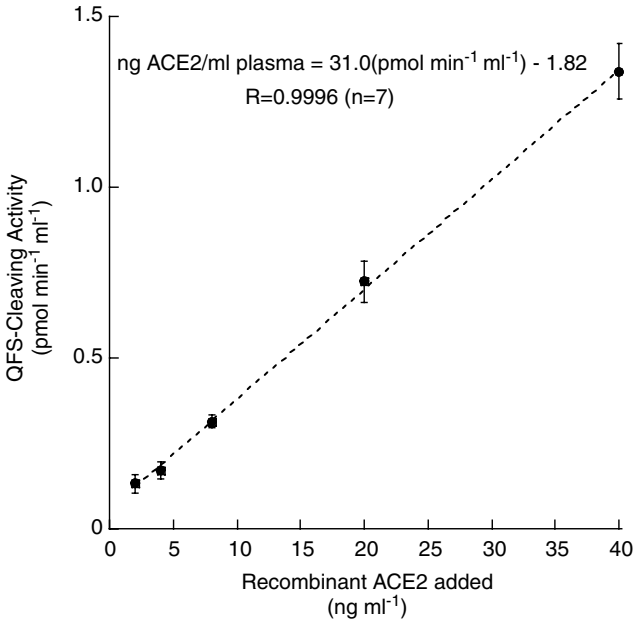

Figure 3. Linear relationship between amount of recombinant ACE2 added to heat-inactivated plasma and the catalytic activity measured following anion exchange extraction
Results are the means ± S.E.M. from 7 independent experiments, and the equation derived by linear regression analysis.

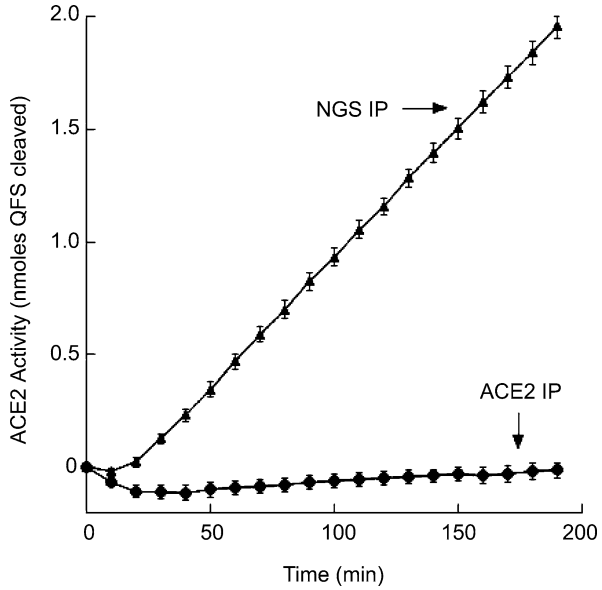

Figure 4. ACE2 immunoprecipitation (IP) of anion exchange extracts of plasma effectively depletes catalytic activity
Results shown are the means ± S.E.M. of 6 plasma samples from separate individuals. NGS, normal goat serum, IgG control.

these samples was completely inhibited by MLN-4760 (102.6 ± 4.9%, $n = 18$). Plasma ACE activity averaged 20.1 ± 2.9 nmol Hip-His-Leu cleaved min^{-1} ml^{-1}, similar to values reported by others (Friedland & Silverstein, 1976; Santos *et al.* 1985; Azizi *et al.* 1997). There was no interaction between age or sex and levels of ACE or ACE2 in plasma.

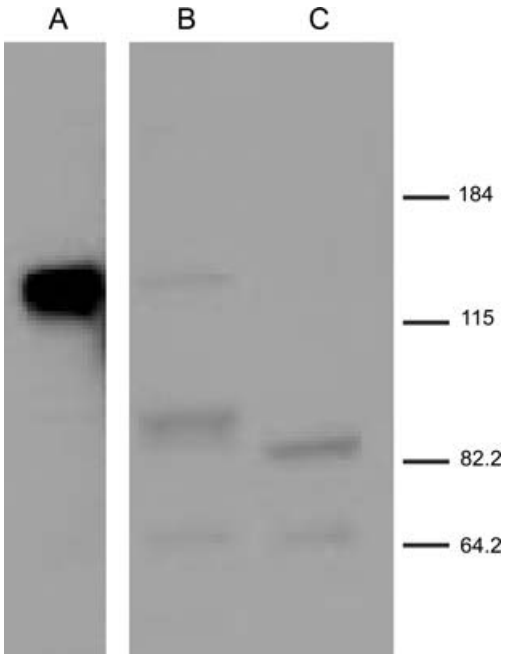

Figure 5. Western blot analysis of immunoreactive ACE2 in human plasma
Lane A is full-length (membrane-bound) human ACE2 purified from transiently transfected HEK293-T cells (300 μg total protein); lane B is derived from human plasma following anion exchange chromatography and concentration (300 μg protein); and lane C is the same as lane B following treatment with PNGase F (4 h).

Discussion

Since its first description in 2000, the research effort into the biochemistry, physiology and pathophysiology of ACE2 has been steadily growing, reflecting its role in cardiovascular regulation as well as its identification as a functional severe-acute respiratory syndrome coronavirus receptor (Li *et al.* 2003). Evidence for an important role for the enzyme in the renin–angiotensin system is accumulating, particularly in the inactivation of Ang II and the formation of Ang(1–7). Several studies indicate that tissue levels of ACE2 are significantly elevated in a range of diseases, suggesting the enzyme may serve to ameliorate some of the detrimental effects of excessive Ang II in these conditions. In the present study, we describe the presence of an endogenous inhibitor of ACE2, which obscures detection of the enzyme by catalytic activity assays. Removal of this inhibitory substance by anion exchange chromatography allowed ACE2 activity to be readily detected in human plasma samples. There have been several previous reports of circulating ACE2 in a number of species; in all cases, levels in normal animals are low, especially in comparison with ACE (Ocaranza *et al.* 2006; Shaltout *et al.* 2007). This agrees with studies of our own in rats showing that plasma levels of ACE2 are normally very low, but are elevated in certain disease states (Herath *et al.* 2007; E. Velkoska, R. Dean & L. M. Burrell, unpublished observations). Interestingly, in these studies, rat plasma did not inhibit recombinant ACE2, and endogenous ACE2 activity could be readily detected (data not shown), suggesting that the inhibitory component may be restricted to humans.

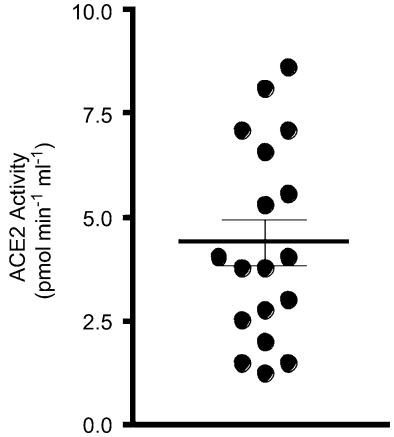

Figure 6. Activity of ACE2 in anion exchange-extracted plasma samples from healthy volunteers ($n = 18$)
Each individual value is represented by a circle, with the mean (thick line) and S.E.M. (thin lines) shown.

Initial characterization of the inhibitory substance suggests that it is small, hydrophilic and cationic. These findings suggest that it might be a divalent cation, which can inhibit metallopeptidases, including ACE (Conroy *et al.* 1978), at high concentrations (Barrett *et al.* 1998). However, other related peptidases, such as neprilysin, thimet oligopeptidase and endothelin-converting enzyme, were at most only modestly affected. Indeed, the closest relative, ACE, is fully active in plasma, suggesting the inhibitor shows a degree of specificity for ACE2. Furthermore, most divalent metal ions circulate in plasma at levels far below those that inhibit metallopeptidases, including ACE2 (R. A. Lew, I. Hanchapola & A. I. Smith, unpublished observations), and often bound to plasma proteins. Indeed, chelation of divalent cations from the 40% acetonitrile fraction of plasma using Chelex-100 resin did not diminish inhibition of ACE2. Other possibilities for the inhibitor currently being considered include basic amino acids or small peptides, possibly even competing substrates as yet unidentified. It is also possible that the levels of this inhibitor may vary, either in a regulated fashion or in disease; modulating the level of circulating inhibitor may allow for the fine control of the activity of both plasma and endothelial ACE2.

The shedding of ACE2 from cells in culture has recently been demonstrated by Lambert *et al.* (2005), who suggest that the phorbol ester-stimulated cleavage of the enzyme from the cell surface is mediated by tumour necrosis factor-α (TNF-α)-converting enzyme (TACE, also called ADAM17), a widely expressed metalloprotease implicated in the cleavage/secretion of a number of membrane-bound proteins, including TNF-α and EGFR (Epidermal growth factor receptor) ligands (Blobel, 2005). Other metalloproteases which are responsible for the basal or unstimulated release of ACE2 have not yet been identified, although they probably also belong to the ADAM (*a* *d*isintegrin *a*nd *m*etalloprotease) family. The present study, together with that of Rice *et al.* (2006) in plasma and our previous work in urine (Warner *et al.* 2005; Lew *et al.* 2006), suggest that shedding also occurs *in vivo* in humans. Western blot analysis not only confirmed the very low levels of ACE2 present in plasma, undetectable without prior extraction by anion exchange and concentration, but also indicated that most of the immunoreactive ACE2 is smaller than the full-length enzyme, and thus is likely to result from proteolytic cleavage (Fig. 5). Whether this is also true in disease states such as myocardial infarction, where tissue necrosis may lead to the release of membrane-bound ACE2, remains to be investigated.

Angiotensin-converting enzyme activity was originally discovered in plasma over 50 years ago (Skeggs *et al.* 1954), but it is generally believed that the circulating enzyme plays little, if any, significant role in angiotensin metabolism relative to tissue ACE, which represents more than 90% of the total ACE content of the body (Ng & Vane, 1967; Xiao *et al.* 2004). Overall, ACE2 expression is much lower and with a more restricted distribution than its homologue, and this is reflected in normal plasma levels at least 10-fold lower than ACE (Alhenc-Gelas *et al.* 1983). From our initial observations regarding the presence of an ACE2 inhibitor in plasma, it appears unlikely that circulating ACE2 contributes significantly to peptide metabolism in the normal situation. However, given the marked increase in tissue levels of ACE2 as a consequence of disease, circulating levels may rise sufficiently to overcome any inhibition, with a subsequent increase in the degradation of angiotensin within the circulation. Indeed, one potential function of the inhibitor may be to counteract the effect of large increases in both plasma and membrane-bound ACE2 in certain diseases, which would otherwise lead to vasodilatation and hypotension. Although tissue ACE2 may not be affected by the inhibitor in plasma, the same may not be true of the enzyme residing on vascular endothelial cells. Further work is necessary to determine the catalytic activity of both circulating and membrane-bound ACE2 in the intact vasculature and the effect of the plasma inhibitor on the increased levels of enzyme that occur with disease.

Analysis of plasma from healthy volunteers indicates a normally low level of circulating ACE2, with measured levels falling within a fairly tight range (1.31–8.69 pmol substrate cleaved $\mathrm{min}^{-1}\,\mathrm{ml}^{-1}$; Fig. 6). A recent family study of circulating ACE in 534 subjects reported mean ACE levels of 6.7 $\mathrm{nmol\,l}^{-1}$ (Rice *et al.* 2006). The same study also reported that plasma ACE2 activity was detectable in only 7.5% of subjects (mean, 33 $\mathrm{pmol\,l}^{-1}$; range, 3 to 460 $\mathrm{pmol\,l}^{-1}$; $n = 40$). These subjects tended to be older and have higher waist-to-hip ratios, blood pressure, fasting glucose levels and other cardiovascular risk factors. Furthermore, half of these subjects had at least one other family member with detectable levels of ACE2. These observations suggest that plasma ACE2 activity levels are typically low, as we have found in the present study, but may be increased in individuals with underlying cardiovascular or other diseases, or with a genetic predisposition to shedding of the enzyme.

Perspectives

Given our previous findings that ACE2 expression is markedly upregulated in the heart following myocardial infarction (Burrell *et al.* 2005) and in the cirrhotic liver (Paizis *et al.* 2005), we have begun to examine plasma ACE2 levels in these patients. Preliminary results suggest that plasma ACE2 is indeed elevated in these conditions, as well as in rat models of chronic liver disease (Herath *et al.* 2007; R. A. Lew, J. S. Lubel, C. Herath, L. M. Burrell, P. W. Angus & A. I. Smith, unpublished observations) and acute renal failure (E. Velkoska, R. Dean & L. M. Burrell, unpublished observations). Thus the appearance of ACE2 in plasma

may serve as a biomarker of pathologies where increased tissue ACE2 expression is observed. Future studies aimed at determining the relationship between plasma ACE2 levels and myocardial or liver disease are currently underway.

References

Alhenc-Gelas F, Weare JA, Johnson RL Jr & Erdos EG (1983). Measurement of human converting enzyme level by direct radioimmunoassay. *J Lab Clin Med* **101**, 83–96.

Azizi M, Ezan E, Nicolet L, Grognet J-M & Menard J (1997). High plasma level of *N*-acetyl-seryl-aspartyl-lysyl-proline: a new marker of chronic angiotensin-converting enzyme inhibition. *Hypertension* **30**, 1015–1019.

Barrett AJ, Rawlings ND & Woessner JF, eds. (1998). *Handbook of Proteolytic Enzymes*, 1st edn. Elsevier, London.

Blobel CP (2005). ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* **6**, 32–43.

Burrell LM, Risvanis J, Kubota E, Dean RG, MacDonald PS, Lu S, Tikellis C, Grant SL, Lew RA, Smith AI, Cooper ME & Johnston CI (2005). Myocardial infarction increases ACE2 expression in rat and humans. *Eur Heart J* **26**, 369–375.

Conroy JM, Hartley JL & Soffer RL (1978). Canine pulmonary angiotensin-converting enzyme. Physicochemical, catalytic and immunological properties. *Biochim Biophys Acta* **524**, 403–412.

Dales NA, Gould AE, Brown JA, Calderwood EF, Guan B, Minor CA, Gavin JM, Hales P, Kaushik VK, Stewart M, Tummino PJ, Vickers CS, Ocain TD & Patane MA (2002). Substrate-based design of the first class of angiotensin-converting enzyme-related carboxypeptidase (ACE2) inhibitors. *J Am Chem Soc* **124**, 11852–11853.

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE & Acton S (2000). A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. *Circ Res* **87**, E1–E9.

Donoghue M, Wakimoto H, Maguire CT, Acton S, Hales P, Stagliano N, Fairchild-Huntress V, Xu J, Lorenz JN, Kadambi V, Berul CI & Breitbart RE (2003). Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins. *J Mol Cell Cardiol* **35**, 1043–1053.

Douglas GC, O'Bryan MK, Hedger MP, Lee DKL, Yarski MA, Smith AI & Lew RA (2004). The novel angiotensin converting enzyme homolog, ACE2, is selectively expressed in adult Leydig cells of the testis. *Endocrinology* **145**, 4703–4711.

Ferrario CM, Trask AJ & 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.

Friedland J & Silverstein E (1976). A sensitive fluorimetric assay for serum angiotensin-converting enzyme. *Am J Clin Pathol* **66**, 416–424.

Hamming I, Timens W, Bulthuis ML, Lely AT, Navis GJ & van Goor H (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* **203**, 631–637.

Herath CB, Warner FJ, Lubel JS, Dean RG, Jia Z, Lew RA, Smith AI, Burrell LM & Angus PW (2007). Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1–7) levels in experimental biliary fibrosis. *J Hepatol* **47**, 387–395.

Hooper NM (1991). Angiotensin converting enzyme: implications from molecular biology for its physiological functions. *Int J Biochem* **238**, 641–647.

Ishiyama Y, Gallagher PE, Averill DB, Tallant EA, Brosnihan KB & Ferrario CM (2004). Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. *Hypertension* **43**, 970–976.

Jordan M, Schallhorn A & Wurm FM (1996). Transfecting mammalian cells: optimisation of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* **24**, 596–601.

Kuruppu S, Reeve S & Smith AI (2007). Characterisation of endothelin converting enzyme-1 shedding from endothelial cells. *FEBS Lett* **581**, 4501–4506.

Lambert DW, Yarski M, Warner FJ, Thornhill P, Parkin ET, Smith AI, Hooper NM & Turner AJ (2005). Tumor necrosis factor-α convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem* **280**, 30113–30119.

Lely AT, Hamming I, van Goor H & Navis GJ (2004). Renal ACE2 expression in human kidney disease. *J Pathol* **204**, 587–593.

Lew RA (2003). HPLC in the analysis of peptide metabolism. In *HPLC of Peptides & Proteins: Methods & Protocols*, vol. 251, *Methods in Molecular Biology*, ed. Aguilar M-I, pp. 275–290. Humana Press, Totowa, NJ, USA.

Lew RA, Warner FJ, Hanchapola I & Smith AI (2006). Characterization of angiotensin converting enzyme-2 (ACE2) in human urine. *Int J Pept Res Ther* **12**, 283–289.

Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H & Farzan M (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454.

Ng KKF & Vane JR (1967). Conversion of angiotensin I to angiotensin II. *Nature* **216**, 762–766.

Ocaranza MP, Godoy I, Jalil JE, Varas M, Collantes P, Pinto M, Roman M, Ramirez C, Copaja M, Diaz-Araya G, Castro P & Lavandero S (2006). Enalapril attenuates downregulation of angiotensin-converting enzyme 2 in the late phase of ventricular dysfunction in myocardial infarcted rat. *Hypertension* **48**, 572–578.

Paizis G, Tikellis C, Cooper ME, Schembri JM, Lew RA, Smith AI, Shaw T, Warner FJ, Zuilli A, Burrell LM & Angus PW (2005). Chronic liver injury in rats and humans upregulates the novel enzyme angiotensin converting enzyme 2. *Gut* **54**, 1790–1796.

Parkin ET, Turner AJ & Hooper NM (2004). Secretase-mediated cell surface shedding of the angiotensin-converting enzyme. *Protein Pept Lett* **11**, 423–432.

Rice GI, Jones AL, Grant PJ, Carter AM, Turner AJ & Hooper NM (2006). Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study. *Hypertension* **48**, 914–920.

Santos RA, Krieger EM & Greene LJ (1985). An improved fluorometric assay of rat serum and plasma converting enzyme. *Hypertension* **7**, 244–252.

Shaltout HA, Westwood BM, Averill DB, Ferrario CM, Figueroa JP, Diz DI, Rose JC & Chappell MC (2007). Angiotensin metabolism in renal proximal tubules, urine, and serum of sheep: evidence for ACE2-dependent processing of angiotensin II. *Am J Physiol Renal Physiol* **292**, F82–F91.

Skeggs LT Jr, Marsh WH, Kahn JR & Shumway NP (1954). The existence of two forms of hypertensin. *J Exp Med* **99**, 275–282.

Steer D, Lew R, Perlmutter P, Smith AI & Aguilar MI (2002). Inhibitors of metalloendopeptidase EC 3.4.24.15 and EC 3.4.24.16 stabilized against proteolysis by the incorporation of β-amino acids. *Biochemistry* **41**, 10819–10826.

Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G & Turner AJ (2000). A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* **275**, 33238–33243.

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A & Tummino P (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**, 14838–14843.

Warner FJ, Lew RA, Smith AI, Lambert DW, Hooper NM & Turner AJ (2005). Angiotensin-converting enzyme-2 (ACE2), but not ACE, is preferentially localized to the apical surface of polarized kidney cells. *J Biol Chem* **280**, 39353–39362.

Xiao HD, Fuchs S, Frenzel K, Teng L, Li P, Shen XZ, Adams J, Zhao H, Keshelava GT, Bernstein KE & Cole JM (2004). The use of knockout mouse technology to achieve tissue selective expression of angiotensin converting enzyme. *J Mol Cell Cardiol* **36**, 781–789.

Ye M, Wysocki J, Naaz P, Salabat MR, LaPointe MS & Batlle D (2004). Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? *Hypertension* **43**, 1120–1125.

Zulli A, Burrell LM, Widdop RE, Black MJ, Buxton BF & Hare DL (2006). Immunolocalization of ACE2 and AT_2 receptors in rabbit atherosclerotic plaques. *J Histochem Cytochem* **54**, 147–150.

Acknowledgements

This work was supported by the National Health & Medical Research Council of Australia, via a Project Grant (268914) awarded to A.I.S., L.M.B. and R.A.L., a C. J. Martin Research Fellowship (F.J.W.) and a Principal Research Fellowship (A.I.S.).