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Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme

Dan Harmer*, Maureen Gilbert, Richard Borman, Kenneth L. Clark

Pharmagene Laboratories Ltd., 2 Orchard Rd., Royston, Hertfordshire SG8 5HD, UK

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Abstract ACE 2, a novel homologue of angiotensin converting enzyme, has recently been identified. This study used QRT-PCR to quantitatively map the transcriptional expression profile of ACE 2 (and the two isoforms of ACE) in 72 human tissues. While confirming that ACE 2 expression is high in renal and cardiovascular tissues, the novel observation has been made that ACE 2 shows comparably high levels of expression in the gastrointestinal system, in particular in ileum, duodenum, jejunum, caecum and colon. Therefore, in probing the functional significance of this novel peptidase, some consideration should be given to a role in gastrointestinal physiology and pathophysiology.

Key words: Angiotensin converting enzyme; ACE 2; Expression profile; Human tissue; Gastrointestinal

1. Introduction

Angiotensin converting enzyme (ACE) catalyses the formation of angiotensin II from angiotensin I, thereby playing a key role in the control of cardio-renal function and blood pressure control [1]. Specific inhibitors of this enzyme are one of the most widely prescribed classes of drugs in cardiology and are used in the treatment of hypertension and heart failure. At a molecular level, human endothelial ACE was first cloned in 1988 by Soubrier and colleagues [2] and characterised as a 170-kDa glycoprotein containing two homologous active sites. Subsequently, it was revealed that a shorter testis specific isoform occurs which results from alternative splicing of the same gene [3,4]. In addition, evidence also exists [5] for a further product of alternative splicing of the ACE gene resulting in excision of the exons encoding the transmembrane domain and thereby encoding a soluble form of the enzyme.

More recently, parallel independent publications from two groups [6,7] revealed a novel gene which encodes a previously unidentified homologue of ACE which has been termed ACE 2 [6] or ACEH [7]. In its catalytic domain, this novel enzyme has approximately 42% identical residues with endothelial ACE. Both the groups reporting the discovery of this novel metalloproteinase also made significant strides in the initial characterisation of its activity. Importantly, ACE 2 is insensitive to classical small molecule inhibitors of human endothelial ACE such as captopril, lisinopril and enalaprilat [7]. Thus, if ACE 2 is shown to be of pathophysiological importance, it represents a novel target for medicinal chemistry driven drug discovery. In terms of enzymatic activity, ACE 2 also differs significantly from endothelial ACE in that it does not catalyse the formation of angiotensin II. Indeed, ACE 2 cleaves the C-terminal amino acid of angiotensin II to form angiotensin 1–7 [7,8] suggesting that ACE 2 may act to provide negative feedback regulation on the activity of the renin–angiotensin system. However, it has been shown that ACE 2 can also efficiently cleave the C-terminal residue from several peptides (apelin-13, dynorphin A 1–13) unrelated to the renin–angiotensin system [8]. Both of the groups which independently identified ACE 2 [6,7] used multi-tissue Northern blotting to gain an initial impression of tissue distribution and found that ACE 2 is expressed in human heart, kidney and testis, consistent with a possible role in cardio-renal function. However, further clarification and quantification of the tissue distribution of ACE 2 is important to help highlight the tissues to study to help elucidate the physiological role(s) of this novel enzyme and possible therapeutic target. In the present study, the quantitative and highly sensitive technique of quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) has been applied to study the transcriptional expression profile of ACE-2 (relative to the other known isoforms of ACE) throughout the human body.

2. Materials and methods

Total RNA was extracted from 72 human tissues (three donors/tissue). Tissues were obtained with approval from the appropriate ethics committees and through partnership with medical intermediaries, hospitals and tissue banks and in all cases with the full informed consent of the donor or their next of kin. Prior to RNA extraction, tissues were assessed histologically by a qualified pathologist and only used if characterised as non-diseased. Tissues were chosen to represent major organ systems of the human body. RNA was extracted using TriZol (Invitrogen Life Technologies), a commercially available mixture of organic solvents. The extractions were carried out as detailed in the manufacturer's protocols. Determining the optical density at 260 and 280 nm assessed the concentration and purity of the RNA. RNA was only used in these studies if the ratio between the 260 nm and 280 nm readings was greater than 1.7. All RNA solutions were diluted to 1 μg/μl in nuclease free water containing RNase inhibitor (N808-0119, Applied Biosystems). The quality of the isolated total RNA was assessed by two methods. Firstly the RNA samples were run down denaturing agarose gels, only samples that had intact 18S and 28S ribosomal bands were used for the study. Secondly, it was determined whether the samples were of high enough quality to be

*Corresponding author. Fax: (44)-1763-211555.
E-mail address: dan.harmer@pharmagene.com (D. Harmer).

Abbreviations: QRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ACE, angiotensin converting enzyme

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used in QRT-PCR, this was done by determining whether the samples were able to produce robust amplification of actin and transferrin receptor mRNA transcripts.

Detection of specific mRNAs was carried out by QRT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using previously described methodology [9].

Forward and reverse primers and the fluorogenic probes were designed to the targets of interest using Primer Express software (Applied Biosystems). The sequences of the primer probe sets used are as follows:

ACE 2, accession number AF291820.

Forward: CATTGGAGCAAGTGTTGGATCTT
Reverse: GAGCTAATGCATGCCATTCTCA
Probe: CTTGCAGCTACACCAGTTCCCAGGCA

This primer probe amplifies a 107 bp region of the mRNA and was designed in the same region as the probe that was used in the Northern blot experiments previously described [6].

ACE, accession number M26657. This set is a generic set which picks up both the testicular and somatic form.

Forward: CCGAAATACGTGGAACTCATCAA
Reverse: CACGAGTCCCCTGCATCTACA
Probe: CAGGCTGCCCGGCTCAATGG

This primer probe set amplifies a 68 bp region of the mRNA.

ACE-testicular, accession number X16295. This set is specific for the testicular form.

Forward: ACGGCCCACCAGACATCA
Reverse: TCATATTCCTCCACAAACTTGCTG
Probe: CAGAGCCCAAACCTGGTGACTGATGA

This primer probe set will pick up a 80 bp sequence of the mRNA.

In addition, the following set was used to amplify a 71 bp portion of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript that spans an exon/exon boundary. This transcript is ubiquitously expressed and was used as an internal positive control within the assay.

Forward: GAAGGTGAAGGTCGGAGTCAAC
Reverse: CAGAGTTAAAAGCAGCCCTGGT
Probe: TTTGGTCGTATTGGGCGCCT

All the probes were quenched with carboxytetramethyl rhodamine and all the target probes, with the exception of GAPDH, were labelled with the fluor 6-carboxyfluorescein, GAPDH used the fluor VIC.

The primer/probe sets were homology searched to ensure that they were specific using an NCBI BLAST search.

3. Results

The quantitative expression map for ACE, ACE testicular and ACE 2 across 72 human tissues is shown in Fig. 1. As anticipated, the testicular form of ACE is expressed almost exclusively in the testis (54 000 copies) with trace expression (<10 copies) in jejunum, pancreas and spleen. The expression map for ACE demonstrates a widespread and ubiquitous distribution. Expression is particularly high in ileum, jejunum, duodenum, testis, lung, pulmonary blood vessels and prostate. ACE 2 is also expressed in many tissues but shows a less ubiquitous profile than ACE. ACE 2 is highly expressed in testis, renal and cardiovascular tissues and notably also in tissues from the gastrointestinal system including ileum, duodenum, jejunum, caecum and colon. Expression of ACE 2 was highest in ileum (copy number = 282 000) of all the 72 tissues evaluated. Expression of ACE 2 was limited in central nervous system and lymphoid tissues.

While previous observations [6,7] suggested that ACE 2 should be highly expressed in cardio-renal tissues, the high expression of ACE 2 in gastrointestinal tissues was unanticipated. Since the initial body-wide expression profiling was restricted to three donors/tissue, experiments were completed in key tissues (ileum, duodenum, caecum, colon, kidney, (cortex, pelvis, medulla) and heart left ventricle) from a further three donors to confirm the initial observations. The results (Fig. 2) confirm that ACE 2 is expressed at high levels in gastrointestinal and cardio-renal tissues. Statistical analysis indicates that while there was no significant difference in expression between ileum and duodenum, ACE 2 expression in ileum and duodenum was significantly higher than in the cardio-renal tissues.

All samples had high GAPDH mRNA expression ($>10\,000$ copies) indicating that they were of high quality.

4. Discussion

The discovery in 2000 of ACE 2, a novel homologue of ACE, has raised significant interest and many laboratories are now working to elucidate the full physiological and pathophysiological significance of this novel enzyme. To that end, in this study we have used the highly sensitive technique of QRT-PCR to determine the level of expression of ACE, ACE testicular and ACE 2 in a wide range of human tissues.

Using QRT-PCR, the human tissue expression profile obtained for somatic and testicular forms of ACE is consistent generally with published data on the expression and physiological significance of these enzymes. Thus, as previously reported [4], the testicular form of ACE is expressed almost exclusively in the testis with trace expression in jejunum, pancreas and spleen. The trace expression that is seen in jejunum, pancreas and spleen is a real signal and cannot be from any contaminating DNA as the primer probe set spans an exon/exon boundary and as such will not work on genomic DNA. This demonstrates the exquisite sensitivity of the technique and is the first observation to our knowledge of the testicular ACE isoform outside of the testis.

For somatic ACE, the primer probe set was a generic set, which detects both the testicular and somatic forms of the enzyme. The expression map for ACE demonstrates a widespread distribution with high expression in cardio-renal tissues, consistent with the known physiological importance of this enzyme. Expression of ACE is also high in ileum, jejunum, duodenum, testis, lung, pulmonary blood vessels and prostate. These results are in concordance with previous studies, which have assessed the expression and activity of ACE in human tissue [10,11].

Having used QRT-PCR to demonstrate that the expression patterns of somatic and testicular ACE are consistent with previous findings, we moved on to study the expression profile of ACE 2 across 72 human tissues. We were particularly interested in looking at ACE 2 as it may be a potential therapeutic target for drug discovery.

QRT-PCR demonstrates that ACE 2 is expressed in many tissues but shows a less ubiquitous expression profile than ACE. ACE 2 is highly expressed not only in testis, renal and cardiovascular tissues, but surprisingly also in tissues from the gastrointestinal system including ileum, duodenum, jejunum, caecum and colon. Its expression in central nervous system and lymphoid tissues was moderate. The present data are consistent with previous observations [6,7] suggesting that ACE 2 is highly expressed in cardio-renal tissues, and with

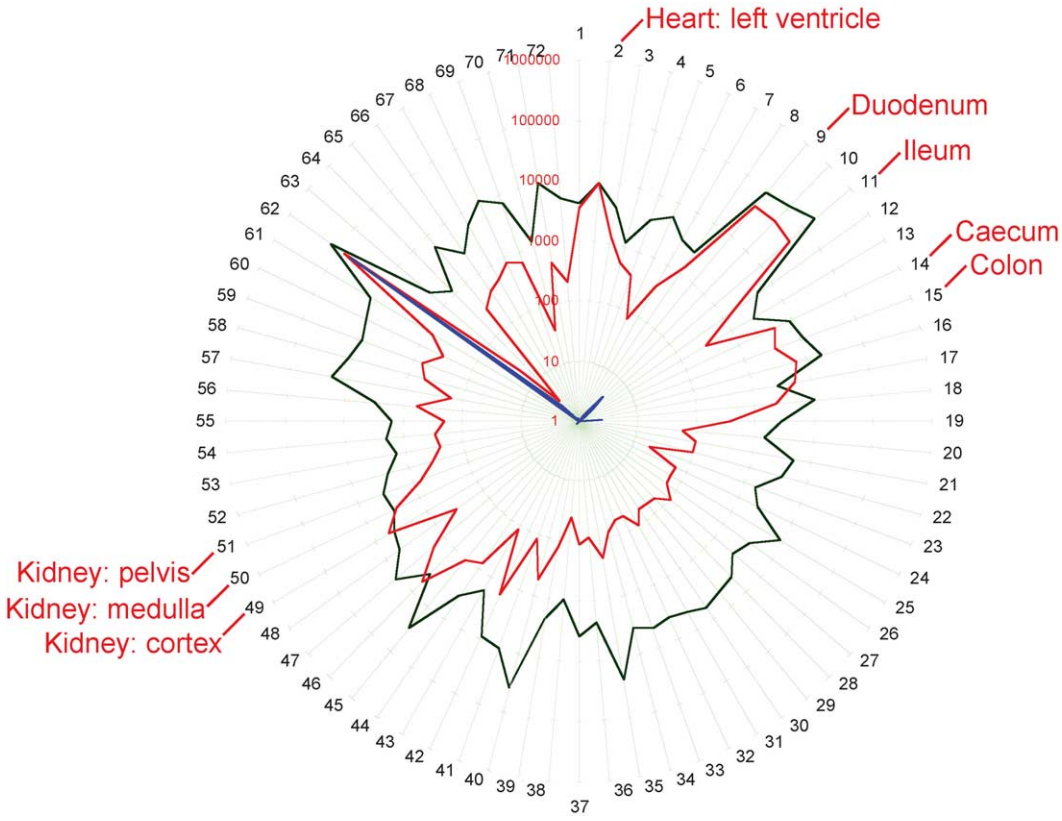

Fig. 1. Stellar plot illustrating the mRNA copy number in logarithmic form for ACE (black), ACE 2 (red) and ACE testicular (blue) in 72 human tissues. Each point represents the geometric mean copy number from determinations in three donors. Gene copy number increases logarithmically moving from the centre to the periphery of the circle. The tissues used are: 1. heart: left atrium; 2. heart: left ventricle; 3. blood vessel: coronary artery; 4. oesophagus; 5. stomach: fundus; 6. stomach: body; 7. stomach: antrum; 8. stomach: pyloric canal; 9. duodenum; 10. jejunum; 11. ileum; 12. adipose: omental ileum; 13. blood vessel: mesenteric (colon); 14. caecum; 15. colon; 16. rectum; 17. gallbladder; 18. pancreas; 19. liver: parenchyma; 20. brain: cerebellum; 21. brain: hippocampus; 22. brain: locus coeruleus; 23. brain: medulla oblongata; 24. brain: amygdala; 25. brain: caudate; 26. brain: hypothalamus anterior; 27. brain: hypothalamus posterior; 28. brain: cortex: cingulate anterior; 29. brain: cortex: cingulate posterior; 30. brain: cortex: frontal-lateral; 31. brain: cortex: frontal-medial; 32. brain: cortex: occipital; 33. brain: cortex: parietal; 34. brain: cortex: temporal; 35. brain: nucleus accumbens; 36. brain: substantia nigra; 37. brain: dorsal raphe nucleus; 38. spinal cord; 39. dorsal root ganglion; 40. pineal gland; 41. pituitary gland; 42. blood vessel: choroid plexus; 43. blood vessel: cerebral: middle cerebral artery; 44. trachea; 45. lung: parenchyma; 46. lung: bronchus: primary; 47. lung: bronchus: tertiary; 48. blood vessel: pulmonary; 49. kidney: cortex; 50. kidney: medulla; 51. kidney: pelvis; 52. blood vessel: renal; 53. ureter; 54. bladder; 55. bladder: trigone; 56. ovary; 57. fallopian tube; 58. uterus: myometrium; 59. uterus: cervix; 60. prostate; 61. vas deferens; 62. testis; 63. spleen: parenchyma; 64. cell: blood mononuclear; 65. lymph gland: tonsil; 66. muscle: skeletal; 67. skin: foreskin; 68. adrenal gland; 69. thyroid gland; 70. umbilical cord; 71. placenta; 72. breast.

recent data supporting an important role of ACE 2 as a regulator of cardiac function [12]. However, our data are in contrast to those reported in an earlier study in which expression of ACE 2 in gastrointestinal tissues was reported to be low [6]. We have shown for the first time significant expression of ACE 2 in the small intestine, an observation previously missed using Northern blotting technique [6,7]. On the basis of these interesting initial findings, we decided to expand the observations to six donors for the key cardiac, renal and gastrointestinal tissues of interest. The data for the six donors confirmed the observation that ACE 2 is highly expressed in gastrointestinal tissue. Statistical analysis of these data showed that the expression of ACE 2 in ileum and duodenum was significantly higher than in any other tissue studied. It is not clear why the current data differ from those of previous workers [6,7] who, using a Northern blotting approach, had at best detected low levels of ACE 2 mRNA in gastrointestinal tissues. It seems unlikely that it reflects donor variation, in the light of the consistency of the high levels detected from samples from six independent donors in the present study. We can therefore only assume that the failure of the previous studies [6,7] to identify high levels of ACE 2 gene expression in gastrointestinal tissues reflects the quality of the RNA used. It is our experience that for tissues obtained from the gastrointestinal tract in particular, short postoperative and post mortem delay times are critical if one is to avoid tissue deterioration and loss of mRNA integrity (unpublished observations). One limitation of the current study is that we have not demonstrated cellular localisation of ACE 2 or confirmed that there is ACE 2 protein present. It would therefore be beneficial to carry out in situ hybridisation and immunohistochemistry studies in the gastrointestinal tissues.

In conclusion, this study demonstrates the utility of QRT-PCR to assess the expression of mRNA transcripts in a wide range of human tissues. High expression of ACE 2 was seen in cardio-renal tissues, in agreement with previous results. However, the identification of ACE 2 at high levels in gastrointestinal tissues is a novel finding and suggests further experiments are warranted to evaluate the role for ACE 2 in gastrointestinal physiology and pathophysiology.

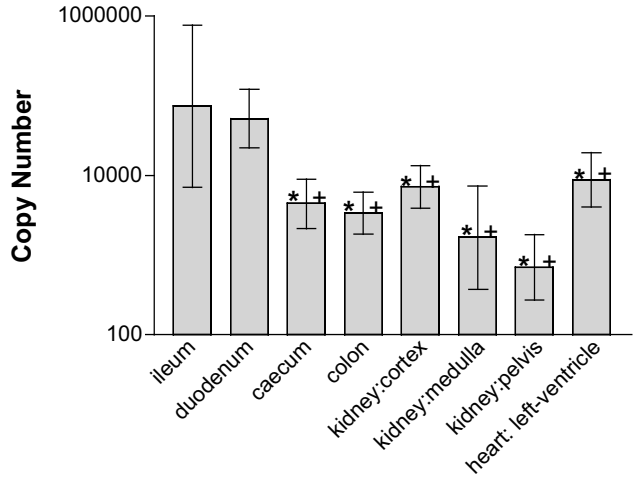

Fig. 2. Quantitative expression profile of ACE 2 in human gastrointestinal, renal and cardiovascular tissues. Values are mean copy number ± 95% ($n = 6$). $^{*}P < 0.05$, statistically significant difference when compared to ileum, $^{+}P < 0.05$ when compared to duodenum (one way analysis of variance followed by Newman–Keuls post hoc test).

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