Selective downregulation of ET\textsubscript{A} receptor mRNA in renal transplant recipients on cyclosporin A revealed by quantitative RT-PCR

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Abstract Despite intensive investigation, a pathophysiological role for the endogenous vasoconstrictor peptide endothelin (ET) remains elusive. The kidney is particularly sensitive to the effects of ET, which are mimicked by the administration of cyclosporin A (CsA), and animal models suggest a role for ET in the vasoconstrictive effects of CsA. Using a recently validated novel fluorescent quantitative RT-PCR assay to enable the direct study of human renal biopsies, we have quantified mRNA for the two known ET receptor subtypes ET\textsubscript{A} and ET\textsubscript{B} in cortical tissue from three groups of patients: renal transplant recipients on CsA (n = 7), those with native renal disease (n = 5) and normal controls (n = 7). Median mRNA levels (amol/μg total RNA) were 0.024, 0.17 and 0.2 respectively for ET\textsubscript{A} and 0.57, 0.64 and 0.96 for ET\textsubscript{B}. These values indicate significant downregulation of ET\textsubscript{A} (P = 0.003) but not ET\textsubscript{B} (P = 0.104) mRNA in the transplant group. These results provide the first demonstration of a perturbation in the human ET system at tissue level in a pathophysiological situation, and suggest that the deleterious renal vasoconstrictor effects of CsA might be ameliorated by selective ET\textsubscript{A} receptor antagonism in the future. This study also illustrates the feasibility of ex vivo analysis of human diagnostic material at the molecular level.

Key words: cyclosporin A; endothelin receptors; human kidney; quantitative RT-PCR

Introduction

The endothelins (ET), whose profound and prolonged vasoconstrictor properties were first described in 1988 [1], exert their effects via two G-protein-coupled receptor subtypes, ET\textsubscript{A} and ET\textsubscript{B}. However, in vitro and in vivo studies have shown significant differences between species both in the receptor subtype mediating vasoconstriction and in the distribution of these receptors. Thus, while ET\textsubscript{A} is the subtype responsible for constriction in the vasculature of humans [2], dog [3], rabbit [4] and pig [5], there is a significant ET\textsubscript{B} component in rat [6]. We have also demonstrated in previous autoradiographic studies the different distribution of ET receptors, particularly in the kidney, and most markedly in the absence of high density intraglomerular binding in humans compared to rat [7].

The ET receptors in the cardiovascular system are in overwhelming majority of the ET\textsubscript{A} subtype, whereas the kidney is one of the richest sites for ET\textsubscript{B} receptors, which are largely non-vascular. The function of the latter is unknown, but observed changes in sodium and water balance after administration of exogenous ET-1 [8] suggest a possible homeostatic role.

Despite intense investigation, a pathophysiological role for the ET system remains to be defined in any species. However, animal studies have suggested that the deleterious renal effects of the potent immunosuppressive agent cyclosporin A (CsA) might be mediated through ET. Bolus injection of CsA in rats causes renal vasoconstriction which is seen to occur at the glomerular arteriolar level [9], a fall in glomerular filtration rate (GFR) and raised plasma ET levels [10] which are very reminiscent of the effects of exogenous administration of ET. CsA is also associated with cellular damage [11] which would release ET.

Rat studies in vivo have shown that the deleterious renal effects of acute CsA administration can be blocked both by the administration of anti-ET antibodies [12] and by intravenous pretreatment with the ET\textsubscript{A}-selective antagonist BQ123 [10].

In clinical practice, the two main adverse effects of CsA are hypertension and nephropathy, both of which are thought to be the result of vasoconstriction. Human CsA nephrotoxicity begins as a reversible decline in GFR [13] and renal blood flow with raised renal vascular resistance even at doses insufficient to raise systemic BP [14], but can end in the development of interstitial fibrosis. The ability of renally derived human cells to synthesize ET [15] and the recognition of raised plasma levels in CsA recipients [16] has thus led to the hypothesis that CsA might mediate its renal vascular effects in humans via the ET system.

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In the light of the important species differences outlined above, it is preferable to address the question of potential pathophysiological roles for the ET system in humans by direct investigation of human tissue, where possible in quantitative as well as qualitative studies. However, the availability of suitable material from biopsies is limited by ethical considerations and yields are too small to permit the use of standard molecular techniques such as RNAse protection or Northern blot. We have therefore developed a fully quantitative fluorescent nested reverse transcriptase—polymerase chain reaction (RT-PCR) assay [17] for the analysis of ET<sub>A</sub> and ET<sub>B</sub> mRNA in diagnostic renal biopsies weighing only a few milligrams.

The prediction was made that, in view of the predominantly vascular distribution of the ET<sub>A</sub> subtype [7], quantitative analysis of ET<sub>A</sub> and ET<sub>B</sub> mRNA would be more likely to reveal changes in the former in the context of CsA therapy. In this study, renal material from three groups of patients was compared: renal transplant recipients on CsA, those with native renal disease and those with histologically normal kidneys.

Subjects and methods

Materials

All laboratory reagents and chemicals were of analytical grade and were obtained from Advanced Protein Products Ltd, Brierley Hill, West Midlands; Aldrich Chemical Co. Ltd, Gillingham, Dorset; Amersham International plc, Amersham, Bucks; BDH Laboratory Supplies, Poole, Dorset; Dako Ltd, High Wycombe, Bucks; Fison’s plc, Loughborough, Leics; Gibco-BRL, Uxbridge, Middx; Sigma Chemical Co., Poole, Dorset. All enzymes were obtained from HT Biotechnology, Cambridge. dNTPs and oligo(dT) were from Pharmacia Ltd, Milton Keynes. 6-FAM (5-carboxyfluorescein)-labelled phosphoramidite was made at the 5' end of primers C and E. These primers were confirmed their identity as human ET<sub>A</sub>- and ET<sub>B</sub>-encoding products cloned and sequenced on both strands. This confirmed of these primers has been reported previously [19], and the common external and specific internal primer pairs for ET<sub>A</sub> and ET<sub>B</sub> receptor cDNA have been designed using published sequences. Primer sequences are shown in Table 2.

RNA extraction

A modification of the single-step guanidinium isothiocyanate method of Chomczynski and Sacchi [18] using reduced volumes and extended precipitation time was employed as specimens weighed 2–10 μg. The final RNA pellet was resuspended in 20 μl sterile water, and up to half used for accurate spectrophotometric quantification.

Primers

Common external and specific internal primer pairs for ET<sub>A</sub> and ET<sub>B</sub> receptor cDNA have been designed using published sequences. Primer sequences are shown in Table 2. The use of these primers has been reported previously [19], and the products cloned and sequenced on both strands. This confirmed their identity as human ET<sub>A</sub>- and ET<sub>B</sub>-encoding fragments spanning exon—intron junctions of sizes 299 and 428 bp, respectively.

For fluorescent PCR product detection, substitution of a 6-FAM (5-carboxyfluorescein)-labelled phosphoramidite was made at the S’ end of primers C and E. These primers were purified by high-pressure liquid chromatography (HPLC) and stored in the dark at −20°C.

Table 1. Comparative characteristics of three patient groups, given as medians (iqr)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 7)</th>
<th>Tx (n = 7)</th>
<th>Native (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67 (48–73)</td>
<td>66 (33–53)</td>
<td>66 (28–67)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>5:2</td>
<td>6:1</td>
<td>5:0</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>106 (90–115)*</td>
<td>283 (247–854)</td>
<td>222 (125–679)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>140 (130–150)</td>
<td>150 (140–150)</td>
<td>145 (122–150)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>80 (80–85)</td>
<td>85 (80–85)</td>
<td>85 (72.5–92.5)</td>
</tr>
<tr>
<td>Drugs (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triple Rx</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Prophylaxis</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Antihypertensive</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Analgesic</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Insulin</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*P<0.03 vs either biopsy group. No other comparisons had P<0.1. Triple Rx = prednisolone, azathioprine and CsA. Prophylaxis = amphotericin, cotrimoxazole and ranitidine. Antihypertensive = nifedipine and/or diuretics.
Sequences are listed in Table 2. Relevant restriction sites, T3 promoter sequences are arranged on either side of a 580 bp ‘stuffer’. Primer B) and specific internal (C, D for ET A; E, F for ET B) primer.

Fig. 1. Structure of synthetic RNA standard. Common external (A, B) and specific internal (C, D for ET A; E, F for ET B) primer sequences are arranged on either side of a 580 bp ‘stuffer’. Primer sequences are listed in Table 2. Relevant restriction sites, T3 promoter site and 3’ poly-A tail are shown.

Construct cRNA synthesis

To produce a cRNA standard, a plasmid was prepared as previously described [17] into which double-stranded oligonucleotide sequences comprising the 5’ and 3’ primer sequences for both ET A and ET B reactions had been cloned in a directional manner between KpnI and HindIII sites. The resulting material contained a 580 bp stuffer segment providing an overall product size difference of about 300 bp. The terms ‘standard’ and ‘construct’ are used interchangeably. Its important features are shown in Fig. 1.

Mixed cDNA synthesis and quantitative PCR

These reactions were carried out as previously reported for this system [17]. Where less than 1.5 µg of tissue RNA were available for quantitative PCR, construct was added at a concentration of 25 pg/ml to maintain an approximate similarity of specific RNA. cDNA synthesis and all subsequent manoeuvres thus applied to both sample and standard in the same tube.

For the first round of PCR, 30 µl of reaction mix overlaid with light mineral oil contained 1 ml mixed cDNA, PCR buffer (10 mM Tris, 50 mM KCl, 1.5 mM MgCl2), 200 uM dNTPs, 1 µM primers A and B, and 1.5 U Taq polymerase. The amplification profile was (95°C, 40°C, 72°C each for 0.5 min) x 20 cycles, preceded by 1.5 min at 95°C and followed by 3 min at 72°C. PCR in the absence of cDNA was always used as a negative control.

For the second round, 20 µl of a similar reaction mix but containing either primers C and D or E and F were used. The final concentration of fluorescently labelled primers C or E was 0.2 µM. Light was excluded where possible. One µl of first round product was transferred as template to the first second-round tube and then six serial 1 in 2 dilutions made. The amplification profile was (95°C, 65°C, 72°C each for 0.5 min) x 24 cycles for ET A or 23 cycles for ET B.

Product sizes were 299 bp for ET A, 628 bp for construct ET A, and 674 bp for construct ET B. All PCR products were kept in the dark at −20°C.

PCR product electrophoresis and quantification

Aliquots of the second-round products were diluted in GS loading buffer (Applied Biosystems). Four µl volumes were loaded onto a 0.4-mm thick, 6% non-denaturing polyacrylamide gel and electrophoresed using an ABI 373A DNA sequencer equipped with Genescan 1.1 software. Peak areas of fluorescence intensity were used to construct sample and standard curves for every assay, and the ratio of sample to standard RNA calculated using a minimum of four points on each occasion.

Statistical analysis

The individual values for ET A and ET B mRNA from different tissues in each of three groups (‘normal’, ‘nat’ and ‘tx’) were tested for normality of distribution using the Shapiro-Wilk W test. Thereafter, all results were expressed as median and interquartile range (iqr). The Mann-Whitney U-test was used to test for differences in mRNA levels between diagnostic groups. The Kruskal–Wallis (K-W) test was applied to compare overall results for ET A or ET B. P<0.05 was accepted as significant. Other parameters (plasma creatinine, age and blood pressure) were each also correlated between groups by K-W testing.

Results

Patients and biopsies

Seven biopsies were obtained from patients with renal transplants [six male, one female; median age 46 years (iqr 33–53)]. None was a smoker. All were receiving triple immunosuppressive therapy (prednisolone, azathioprine and CsA) and two were on the usual infection prophylaxis regimen in use (cotrimoxazole, amphotericin mouthwash, ranitidine). These biopsies were performed because of clinical concern over either primary non-function (one patient) or graft dysfunction not due to vascular failure (i.e. graft Doppler ultrasound showed patent renal vessels in all patients). Median serum creatinine was 283 µmol/l (iqr 247–854). Histological appearances were of mild cellular rejection in three, early humoral rejection in one and were normal in two. The other showed acute tubular necrosis (ATN). No patient had histological evidence of either cyclosporin toxicity or medial disruption.

Five biopsies came from patients investigated for native renal disease (Table 1) with median age 46 years (iqr 28–67); two were smokers. Mean serum creatinine

Table 2. Primer sequences for PCR amplification of ET receptor mRNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ET A, ET B forward (external)</td>
</tr>
<tr>
<td>B</td>
<td>ET A, ET B reverse (external)</td>
</tr>
<tr>
<td>C</td>
<td>ET A forward (internal)</td>
</tr>
<tr>
<td>D</td>
<td>ET B reverse (internal)</td>
</tr>
<tr>
<td>E</td>
<td>ET B forward (internal)</td>
</tr>
<tr>
<td>F</td>
<td>ET B reverse (internal)</td>
</tr>
</tbody>
</table>

Table 1. Patients and biopsies

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>M</td>
<td>Native</td>
<td>Transplant</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>Native</td>
<td>Native</td>
</tr>
<tr>
<td>53</td>
<td>M</td>
<td>Renal</td>
<td>Transplant</td>
</tr>
<tr>
<td>46</td>
<td>M</td>
<td>Native</td>
<td>Native</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>Renal</td>
<td>Transplant</td>
</tr>
</tbody>
</table>

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ETα mRNA downregulation in CsA-treated renal transplant biopsies

of 222 μmol/l was not significantly different from the transplant group (P = 0.42). Histologically, four had evidence of mild focal fibrosis, of whom one also had hypertensive vascular disease. The fifth had membranous nephropathy.

Seven normal kidney cortex samples analysed came from histologically normal tissue in those undergoing nephrectomy for non-obstructing tumours. Median serum creatinine was 106 μmol/l and was significantly lower than either of the biopsy groups. Blood pressure and age were not significantly different. Table 1 illustrates the comparative clinical characteristics of all three groups.

ETα mRNA levels

As displayed in Fig. 3a and Table 3, analysis of the three groups as a whole showed a highly significant decrease in the amounts of ETα mRNA present in transplant biopsies compared with either native biopsies or normal kidneys. Mann–Whitney testing yielded P < 0.005 vs normals and P < 0.006 vs native biopsies. There was no difference between native and normal kidneys (P = 0.75). The Kruskal–Wallis H statistic was 11.55 giving P = 0.003.

ETβ mRNA levels

For ETβ, there were no statistically significant differences between groups. Kruskal–Wallis testing of these data found P = 0.104. Data are shown in Fig. 3b and Table 3.

These data were reanalysed separately after removal of samples Tx1 (chronic vascular rejection) or Tx6 (severe ATN) or Nat4 (severe hypertensive vascular disease) from the analysis, and a significant difference was found (Kruskal–Wallis H statistic = 8.44, P = 0.015).

Fig. 2. Method for quantitative fluorescent nested RT-PCR. A known amount of synthetic standard RNA is mixed with the RNA under investigation. The standard contains sequences recognized by the same primer pairs as the sample (here I to IV) but the products are of different lengths. cDNA synthesis and all subsequent procedures apply to both sample and standard in the same tube. For the second round a 2-fold dilution series is made using an aliquot of first-round product. Asterisks indicate 5’-fluorescein primer end-labelling, permitting analysis and quantification of PCR products using an automated sequencer.
disease) in order to determine if these particular diagnoses were responsible for statistically significant skewing of results. In the event, there were no changes in overall significance of the findings. Of note, the small trend towards loss of ET\(_B\) in transplants disappeared (P = 0.14) without affecting ET\(_A\) results (P = 0.008) when Tx6 was excluded.

No significant correlations were found between mRNA levels and either age, BP or creatinine level in any group.

Discussion

The use of this fluorescent quantitative PCR assay allows, for the first time, the fully quantitative analysis of an endogenous receptor system at the mRNA level in ex vivo human biopsy tissue. The results presented show that in the context of renal transplantation and immunosuppression, the ET\(_A\) subtype message is downregulated whereas the ET\(_B\) subtype is not similarly affected. We chose quantitative RT-PCR in order to measure mRNA levels in these tissues since it is not possible with such small amounts of material to carry out ligand binding experiments. However, in larger samples of normal kidney there was a direct correlation between the amount of mRNA (quantified by this method) and the amount of receptor protein (previously measured by ligand binding) [20]. Therefore while direct confirmation is not possible, these results strongly suggest that ET\(_A\) receptor protein is also downregulated.

There are several possible interpretations of these data. Firstly, the downregulation seen could be a direct consequence of CsA therapy in these patients. The well-recognized phenomena of CsA-associated hypertension and impairment of renal function in humans, with up to 25% increase in plasma creatinine levels seen even in those whose CsA levels remain well within the therapeutic range [21], have so far defied functional explanation beyond the knowledge that vasoconstriction is central to these effects. Because the mechanism remains unclear, several studies have attempted different therapies to reverse these chronic CsA administration effects. These include limited success with prostaglandin analogue administration [22, 23], but conflicting results with different calcium channel antagonists nifedipine [24] and felodipine [25]. These pharmacological interventions share the functional effect of vasodilatation, but shed no further definitive light on the underlying pathophysiology. Despite the majority of both transplant recipients and native disease patients receiving similar antihypertensive therapy in our study, downregulation of ET\(_A\) mRNA was observed only in the former group.

By analogy, one could conclude that CsA directly stimulates ET to cause the vasoconstriction in the kidney, and that vascular ET\(_A\) receptors downregulate as part of a negative feedback loop. By such a mechanism, only a small effect on ET\(_B\) might be expected, since most of this subtype is non-vascular [7].

Precedents for regulatory changes in endogenous ligand/receptor systems are widespread, for example the downregulation in \(\beta\)-receptors consequent upon chronic \(\beta\)-agonist therapy in asthma [26]. This explanation is also consistent with the elevated plasma ET levels previously found in humans on CsA, and by Fogo et al.'s protective pre-emptive administration of BQ123 in rats with acute CsA toxicity [10]. However, Davis and colleagues [27] performed a similar study which failed to show protection by BQ123. There were several differences between the two protocols. Fogo's positive results followed the intrarenal administration of BQ123, and noted that a higher dose administered systemically had no effect. Davis administered the antagonist only systemically, in a dose 10 times greater still, and showed no effect. Since systemic venous administration implies passage through the lungs prior to drugs reaching the kidney, it is perhaps not surprising that the renal protection appeared to require local dosing. Another caveat is that differences in study outcome might be expected when CsA is given to those with intact kidneys or after renal transplantation where the kidney is denervated. However, changes in the density of ET receptors in transplanted cardiac tissue have not been found using ligand binding assays (Davenport et al., unpublished observations).

A second related explanation for ET\(_A\) downregulation in this context is that CsA is responsible for direct endothelial toxicity such that ET is released from damaged cells, thereby producing unwanted vasoconstriction and a similar negative feedback via ET\(_A\) on adjacent smooth muscle. A case report [11] documents severe endothelial injury in a CsA-treated patient, and histological features of CsA toxicity do include endothelial disruption. However, none of the biopsies examined here showed this appearance, making this theory less tenable.

A third possible reason for ET\(_A\) downregulation is that CsA is exerting its vasoconstrictive effects through an unrelated mechanism, the kidney's response being to attempt vasorelaxation via ET receptors. This is all the more relevant in the context of renal transplantation since neurogenic mechanisms have been removed. Bantle et al. [28] demonstrated that in those on long-term CsA whose renal function remained stable, other effects such as reduced plasma renin activity occurred, which represents another possible compensatory mechanism. These points may also help to explain why renal allograft recipients seem to be less tolerant of high doses of CsA compared with those receiving other organs. A study in rats, using very high CsA doses in an acute protocol sufficient to cause immediate renal dysfunction [29], suggested that in this animal, total ET receptor density was increased by some 60%. However our patients received long-term titrated doses in the therapeutic range, and in view of the interspecies differences mentioned earlier, direct comparison is not appropriate.

Biopsies were performed in the transplant recipients because of suspected rejection. Several had histological features of early cellular rejection and one of humoral
rejection, both of which affect the vessels. However the changes seen were mild in all cases, without medial disruption, and two were histologically normal. Since varying histological appearances accompany similar results, no trend can be established, but tissue damage itself does not account for these results.

The observed differences in ET$_A$ mRNA levels between native and transplant biopsies make it unlikely that renal dysfunction per se caused changes in the transplant tissue, since median creatinine levels were the same in both groups.

The results presented here demonstrated no significant changes in ET$_B$ mRNA levels. This is in agreement with the observation that little if any of the vasoconstrictor action of ET-1 in the human kidney appears to be mediated via this subtype [2]. Little is known of the function of the largely non-vascular ET$_B$ subtype in humans; possibilities include a clearance role or involvement in the observed changes in sodium and water balance which follow ET administration [8].

Although the precise mechanism by which CsA causes its detectable vasoconstriction in humans remains unclear, the finding of ET$_A$ but not ET$_B$ downregulation in this context provides a good pathophysiological setting for the assessment of newly available orally active ET antagonists. In the absence of a known function for the ET system in any animal, it is evident that the important question of its true pathophysiological role will ultimately be answered by the use of antagonists in a clinical setting. There is therefore an urgent need to identify patients where such compounds, already in Phase I trials, are most likely to be beneficial. Thus further patients such as those described here may be recruited, on the basis of molecular evidence of a perturbation in the ET system, for clinical trials of potentially therapeutically useful agents. The results of this study suggest that therapeutic benefit may be gained from the administration of an ET$_A$-selective agent to minimize the vasoconstrictive effects of CsA.

The results drawn from this study also serve to illustrate the general principle of the feasibility of analysis of human biopsy tissue at the mRNA level. The temptation must be avoided of resorting to single point analysis of samples, because of tube to tube variability [30]. However, in many situations this approach may well suffice, for example when very large changes are expected (for example in viral expression). For accurate quantification it is necessary to establish that the exponential phase of the reaction applies to sample and standard in preliminary assays. The fact that the gradients of standard and sample fluorescent intensity curves were always parallel in the exponential phase confirmed that they were amplifying at the same rate despite their size differences [17]. This technique is of general applicability provided that a suitable cRNA standard can be constructed, and offers the possibility of comparative or serial analyses of tissue from living individuals.

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