Renoprotective differences between perindopril and enalapril in the diabetic hypertensive rat do not reflect glomerular angiotensin-converting enzyme activity

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1. The various angiotensin-converting enzyme inhibitors have structural differences which affect their affinities for the catalytic sites on converting enzyme. We postulated that such differences might result in differences in renoprotective efficacy. We investigated this in the diabetic spontaneous hypertensive rat. We also investigated whether these differences might reflect variations in glomerular or plasma angiotensin-converting enzyme activity.

2. One week after induction of diabetes, rats were started on antihypertensive therapy: enalapril, 10 mg·day⁻¹·kg⁻¹, or perindopril, 4 mg·day⁻¹·kg⁻¹, in the drinking water. After 3 months, the rats were killed, blood samples were taken and tissues were harvested. Angiotensin-converting enzyme activity in isolated glomeruli and plasma was measured by fluorimetric assay. Glomerular protein content was also determined.

3. Urinary protein excretion was significantly lower in perindopril-treated rats than in either controls (P < 0.0005) or enalapril-treated rats (P < 0.05). Glomerular protein content was also lower in perindopril-treated rats (P < 0.05 versus enalapril; P < 0.005 versus control). There was no difference in glomuerlar angiotensin-converting enzyme activity between the two inhibitors although both were lower than control values (enalapril P < 0.025; perindopril P > 0.025). Plasma angiotensin-converting enzyme activity was significantly lower in the perindopril group than in either control (P < 0.005) or the enalapril group (P < 0.01).

4. We conclude that in the spontaneous hypertensive rat with streptozotocin-induced diabetes, perindopril is more effective than enalapril in reducing proteinuria and glomerular protein accumulation. This difference does not result from differences in glomuerlar-converting enzyme activity.

INTRODUCTION

Treatment with angiotensin-converting enzyme (ACE) inhibitors has been shown to slow the rate of progression of diabetic nephropathy in both human subjects [1-4] and experimental animals [5,6]. However, the mechanism(s) of this beneficial effect remain uncertain. It has been postulated that it results from a decrease in glomerular hypertension and/or hyperfiltration [7] and that the pathological changes in the glomerulus are secondary to changes in glomerular haemodynamics [7,8]. Angiotensin II (Ang II) increases efferent arteriolar tone [7,9], thereby causing an increase in filtration fraction, and also promotes cellular hypertrophy by a direct interaction with its AT1 receptor [10]. It has been postulated that the benefits of ACE inhibition may be the result of decreased circulating or renal Ang II concentrations. Alternatively, they have been ascribed to an increase in bradykinin concentration as this peptide acts as a renal vasodilator [11] and may inhibit cellular hypertrophy [12].

ACE is also known to hydrolyse numerous other peptide hormones including luteinizing hormone-releasing factor (LHRF), cholecystokinin octopeptide, substance P, neurotensin and the enkephalins. It has two catalytic sites, termed the C-terminal and N-terminal active sites, which are thought to be substrate specific. Angiotensin I (Ang I) is hydrolysed to Ang II at the C-terminal site. In contrast, bradykinin [1-9] is catabolized to bradykinin [1-7] at both the C-terminal and N-terminal sites. Other substrates such as LHRF and cholecystokinin-8 are thought to be preferentially cleaved by the N-terminal site.

The structural variations among the numerous ACE inhibitors have resulted in differing specificities for the C- and N-terminal active sites. Those ACE inhibitors with hydrophobic side chains, such as perindopril and quinapril, bind principally to the C-terminal site and have little or no affinity for the N-terminal site [13]. In contrast, inhibitors such as enalapril and lisinopril bind with equal efficacy to both active sites [13].

We postulated that these differences in site specificities would influence the efficacy of ACE inhibitors in ameliorating experimental diabetic nephropathy, and that such a difference in efficacy might correlate with variations in measurable ACE activity in plasma or the glomerulus. To investigate this, we compared equi-

Keywords: angiotensin-converting enzyme inhibitors, diabetic nephropathy, glomerular angiotensin-converting enzyme activity, plasma angiotensin-converting enzyme activity.

Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; LHRF, luteinizing hormone-releasing factor.

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potent doses of enalapril and perindopril in the spontaneous hypertensive rat with streptozotocin-induced diabetes.

METHODS

Experimental protocol

Ten-week-old spontaneous hypertensive rats, weighing 110–130 g, were randomized to three experimental groups (n = 16 each group). They were housed four per cage and allowed ad libitum access to a normal rat chow (Doust and Rabbidge, Sydney, Australia) and drinking water. The rats were acclimatized to handling, blood pressure measurement [by tail cuff plethysmography (Narco Biosystems, Sydney, Australia)], drinking water. The rats were acclimatized to hand-made and blood collected via aortic puncture for measurement of electrolytes, creatinine and ACE activity. The kidneys were then removed and immediately snap-frozen in liquid nitrogen and stored at −80 °C until use.

Serum ACE activity

ACE activity was determined in serum by incubating 10 μl of serum with 240 μl of hippuryl-histidyl-leucine [15] at room temperature for 15 min. The reaction was stopped by addition of 0.28 mol/l sodium hydroxide. After incubation with o-phthaldialdehyde the histidyl-leucine generated was measured fluorometrically by comparison with a standard curve. The inter- and intra-assay coefficients of variation were 8.9% and 3.4% respectively.

Glomerular ACE activity

On the day of the experiment, kidneys were thawed at 4 °C and the glomeruli obtained by differential sieving [16]. After resuspension in cold PBS (Oxoid, Melbourne, Australia), the glomerular suspension was divided into two aliquots and washed again. One pellet was resuspended in PBS containing EGTA, 5 mmol/l, and the other in PBS alone. Both were incubated for 45 min at room temperature then washed four times in cold PBS.

For determination of ACE activity 100 μl aliquots of glomerular suspension were incubated with hippuryl-histidyl-leucine (5 mmol/l) at 37 °C for 60 min. Those tubes previously incubated with EGTA had ZnSO₄ (100 μM) included in the assay buffer. The reaction was stopped by addition of 0.28 mol/l sodium hydroxide. The amount of histidyl-leucine generated was determined fluorometrically by comparison with a standard curve as described above. Activity was expressed as nmol·min⁻¹·glomerulus⁻¹.

Validation of method for removal of ACE inhibitors and reconstitution of converting enzyme activity

The method for removal of ACE inhibitors from glomeruli was based upon that described by Kohzuki et al. [17], who demonstrated in membrane preparations that ACE inhibitors could be dissociated from tissue by chelating the zinc ion from the active site. They further demonstrated that enzyme activity could be reconstituted by incubation with zinc to reveal total ACE activity, the combined activity of active (unbound) and previously inhibitor-blocked ACE.

In vitro inhibition. Glomeruli were obtained by differential sieving as described previously, washed by centrifugation in cold PBS and then resuspended in PBS alone or in PBS containing either enalaprilat (10⁻⁸–5 × 10⁻⁶ g/ml) or perindopril (5 × 10⁻⁸–10⁻⁶ mg/ml). After incubation at room temperature for 60 min, ACE activity was assayed by fluorimetric estimation of histidyl-leucine generated by incubation...
of samples with hippuryl-histidyl-leucine. ACE activity in the enalaprilate- and perindoprilate-treated samples varied with inhibitor concentration and ranged between 10 and 60% of that of the untreated controls. This demonstrated that adequate inhibition could be obtained by in vitro incubation to permit validation of methods for inhibitor removal and activity reconstitution.

**ACE inhibitor removal and reconstitution.** Glomeruli were obtained and washed as above and divided into four aliquots. Two aliquots were incubated with enalaprilate (10^{-8} g/ml) in PBS and the other two in PBS alone at room temperature for 60 min. After washing and resuspension, one enalaprilate and one control aliquot were incubated at room temperature with 5 mmol/l EGTA in PBS, pH 7.3 for 45 min. The other enalaprilate and the untreated aliquots were resuspended in PBS and left at room temperature for 45 min.

All four tubes were then washed and the glomeruli were resuspended in phosphosaline buffer containing ZnSO_{4} (100 μmol/l). ACE activity was then measured as described above. This experiment confirmed an average 50–60% inhibition of ACE activity in the enalaprilate tube compared with the untreated control. The EGTA-treated controls showed 100% recovery of activity after preincubation with zinc while the enalapril group exposed to EGTA showed an average recovery to 96–100% of the activity of the untreated non-EGTA-exposed controls. Similar results were obtained after in vitro exposure of glomeruli to 10^{-8} g/ml perindoprilate.

EGTA rather than EDTA was used as the concentration of EDTA required to remove the ACE inhibitor (20 mmol/l) prevented full recovery of activity in response to incubation with ZnSO_{4}. This was demonstrated by a 20–30% decrease in the activity of non-ACE-inhibited glomeruli which were treated with EDTA compared with controls.

**Removal and reconstitution after in vivo ACE inhibition.** Rats were given either perindopril (4 mg/kg) in the drinking water or drinking water alone for 7 days. On day 7, they were anaesthetized as above and the kidneys harvested. Glomerular ACE activity was then measured with and without removal of the ACE inhibitor and reconstitution with ZnSO_{4}. Glomerular ACE activity in the perindopril-treated rats was 132.9 ± 6.2 nmol·min^{-1}·g^{-1} protein before inhibitor removal and 155.7 ± 6.9 nmol·min^{-1}·g^{-1} protein after removal of the perindopril. This was significantly greater than in those rats without perindopril removal (P < 0.025) and in the untreated controls (137.7 ± 6.5 nmol·min^{-1}·g^{-1} protein, P < 0.05).

**Glomerular protein content**

Glomerular concentration in each final suspension was determined by counting in a graticule, of three separate 20 µl aliquots. The protein content of each final suspension was estimated from 100 µl aliquots by the method of Lowry et al. [18]. The glomerular protein content for each rat was then calculated by dividing the glomerular protein concentration by the glomerular concentration for each suspension.

**Statistical analysis**

All values were expressed as means ± S.E.M. Differences within and between groups were assessed by analysis of variance. Individual paired comparisons were made by Student’s t-test using pooled variance estimates when the variances were equivalent and a separate estimate when variances were different (CSS-Statistica). Statistical significance was determined at the P < 0.05 level.

**RESULTS**

**Blood pressure and blood glucose**

Systolic blood pressure was similar in the three groups before induction of diabetes (Figure 1, top). After ACE inhibitor therapy, the blood pressure in both treatment groups was significantly lower than the control group at all time points. Systolic pressure in the perindopril group was somewhat greater than in the enalapril-treated rats throughout, although this difference only attained statistical significance during the second month after induction of diabetes (enalapril, 115.0 ± 3.2 mmHg; perindopril, 126.4 ± 3.7 mmHg; P < 0.025).

Blood glucose control was similar in all groups: controls, 6.7 ± 0.4 mmol/l; enalapril-treated rats, 6.7 ± 0.4 mmol/l, and 6.6 ± 0.3 mmol/l in the perindopril-treated group.

**Creatinine clearance**

There was no significant difference in creatinine clearance between the groups before administration of streptozotocin. After induction of diabetes, creatinine clearance increased in the control group and decreased in both treatment groups (Figure 1, middle). At the end of the first month creatinine clearance in the control group (1.31 ± 0.13 ml·min^{-1}·100 g^{-1}) was significantly greater than in the enalapril- (0.63 ± 0.08 ml·min^{-1}·100 g^{-1}, P < 0.0005) and perindopril-(0.68 ± 0.03 ml·min^{-1}·100 g^{-1}, P < 0.0005) treated groups. The two treatment groups did not differ significantly. During the second and third months after induction of diabetes, the creatinine clearance in the control group remained significantly greater than in both the treatment groups. In the perindopril-treated animals creatinine clearance was significantly lower than in the enalapril-treated animals from the second month (month 2, P < 0.005; month 3, P < 0.01). In both treatment groups, these values at 3 months remained within the normal range for creatinine clearance in the rat [19].
Urban protein excretion

In the control group, urban protein excretion had increased significantly from pre-diabetic levels (2.86 ± 0.28 mg/24 h) by 1 month (16.70 ± 1.34 mg/24 h, P < 0.0005; Figure 1, bottom). It then continued to increase over the study period.

In the enalapril-treated rats proteinuria also increased but to a markedly lesser extent than in the control group. There were significant differences between the control and enalapril groups at month 1 (P < 0.0005), month 2 (P < 0.005) and month 3 (P < 0.005) after induction of diabetes.

Glomerular protein content

The protein content of the glomeruli was significantly different in the three groups (Figure 2). Glomerular protein was greater in controls (68.8 ± 3.1 ng/glomerulus) than in the enalapril- (57.7 ± 4.7 ng/glomerulus, P < 0.05) or perindopril-treated rats (47.7 ± 2.8 ng/glomerulus, P < 0.005). The protein content of the glomeruli in the perindopril-treated rats was also lower than in the enalapril-treated rats (P < 0.05).

Glomerular and serum ACE activity

Serum ACE activity was significantly lower in the perindopril-treated rats (30.8 ± 4.8 nmol·min⁻¹·ml⁻¹) than in both the enalapril-treated group (47.0 ± 5.5 nmol·min⁻¹·ml⁻¹, P < 0.01) or control rats (51.6 ± 3.0 nmol·min⁻¹·ml⁻¹, P < 0.005; Figure 3, top).

There was no significant change in glomerular ACE activity in either of the treated groups after removal of the inhibitor with EGTA and reconstitution with ZnSO₄. Glomerular ACE activity was significantly lower in both enalapril (P < 0.025) and perindopril (P < 0.025) groups than in the control group. Unlike plasma ACE activity there was no significant difference in the glomerular ACE activity between the two treated groups (Figure 3, bottom).

DISCUSSION

This study demonstrates that ACE inhibitors differ in their ability to ameliorate indicators of nephropathy in the spontaneous hypertensive rat with streptozotocin-induced diabetes. Perindopril, an ACE inhibitor which binds preferentially to the C-terminal
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**Figure 3**  Serum and glomerular ACE activity

Top: ACE activity in serum in control (open bar), enalapril- (hatched bar) and perindopril- (solid bar) treated rats 3 months after induction of diabetes. *P < 0.01 perindopril compared with enalapril; **P < 0.005 perindopril compared with control.

Bottom: glomerular ACE activity. For enalapril- and perindopril-treated groups, the left-hand bar represents unblocked ACE activity and the right-hand bar ACE activity after removal of the inhibitor by treatment with EGTA and reconstitution with zinc. *P < 0.025 enalapril compared with control; #P < 0.025 perindopril compared with control.

site and has little or no affinity for the N-terminal site, was more effective than enalapril, which binds to both catalytic sites. Not only was perindopril more efficacious than enalapril in reducing manifestations of diabetic nephropathy, such as proteinuria, it also appears more effective in preventing structural damage within the kidney. Glomerular protein content, which reflects glomerular basement membrane thickness and mesangial area [14], was significantly less in the perindopril-treated group than in the rats that received enalapril.

These structural and functional differences between the two treatment groups do not appear to be due to differences in the abilities of enalapril and perindopril to inhibit glomerular ACE or to prevent induction of this enzyme within the glomerulus. Converting enzyme activity, as measured by cleavage of hippuryl-histidyl-leucine, was similar in both treated groups before removal of the ACE inhibitor by EGTA. Furthermore, in neither group was there significant induction of glomerular ACE in response to therapy. The glomerular ACE activities in both treated groups were similar before and after removal of the inhibitor and reconstitution of the enzyme with zinc. This is in contrast to our acute preliminary study in which ACE activity increased after removal and reconstitution in the perindopril-treated rats. This suggests that chronic ACE inhibition, unlike short-term treatment, results in a decreased rate of synthesis of converting enzyme within the glomerulus. These findings in the glomerulus differ from those reported by Cooper et al. [5] for ACE activity in plasma. They reported that the percentage of ACE activity which was suppressed by perindopril decreased with time, suggesting some induction of ACE synthesis. The difference between the two studies may reflect tissue-specific differences, as plasma ACE is derived from the lung. This concept is supported by the work of Hirsch et al. [20], who showed differences between various tissues in response to ACE inhibition, the kidney showing a decrease while ACE activity increased in the lung.

It is possible that the doses of perindopril and enalapril which were chosen were not equipotent. Although the two doses which were used in this study were equipotent with regard to blood pressure there were differences in serum ACE activity between the two treatment groups. It might be argued therefore that, with respect to their effect on serum ACE activity, the two doses chosen were not equivalent and an effectively higher dose of perindopril was administered. In contrast, however, in the target organ of interest ACE activity was similar in both treatment groups suggesting equal efficacy in terms of tissue effect. The relative roles of serum and glomerular ACE activity in progressive glomerular sclerosis are unresolved but it could be reasoned that glomerular ACE activity is the more important of the two.

The differences in glomerular sclerosis may reflect the differences in glomerular haemodynamics between the groups. However, we have shown previously that when blood pressure and glomerular filtration rate were reduced to a comparable degree by treatment with verapamil and enalapril, there were significant differences in the degree of glomerular sclerosis [14]. This suggested that structural changes within the glomerulus occur not only in response to haemodynamic perturbation but also as a direct effect, possibly the interaction of Ang II with its AT1 receptor causing protein synthesis [10]. The results of the present study, however, suggest that not only C-terminal substrates such as Ang II may be involved in this structural change but also N-terminal substrates. As perindopril has little affinity for the N-terminal site, hydrolysis of N-terminal substrates would continue with perindopril treatment but would be decreased in response to treatment with enalapril. N-terminal substrates, such as LHFR, would therefore be more likely to accumulate in the enalapril-treated group and possibly contribute to the greater degree of structural
change seen in this group evidenced by the difference in glomerular protein. This might be as a direct effect of the peptide or indirectly via sex steroids in the case of LHRF. The latter concept is supported by the work of Bach et al. [21], who demonstrated a decrease in glomerular basement membrane thickness in castrated compared with whole diabetic rats.

We conclude that there are differences in the degree of glomerular sclerosis after treatment with perindopril and enalapril in doses which are equipotent with respect to blood pressure and glomerular but not serum ACE activity. This difference may be related to differences in their structure and binding affinities for the two active sites on the enzyme.

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REFERENCES

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