Modulation of Incipient Glomerular Lesions in Experimental Diabetic Nephropathy by Hypotensive and Subhypotensive Dosages of an ACE Inhibitor

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A glomerular permeability defect occurs early in the course of type 1 diabetes and precedes the onset of microalbuminuria and renal morphological changes. Recently, ACE inhibitors have been shown to prevent loss of glomerular membrane permselective function, but the mechanism of this nephroprotective effect is still being debated. The objective of the present study was to evaluate the effects of hypotensive and subhypotensive dosages of the ACE inhibitor quinapril on the glomerular albumin permeability (P_{ab}) defect in the early phases of experimental diabetes. For the ex vivo study, six groups of male Wistar rats were evaluated for 4 weeks. One group served as a nondiabetic control (C); the other five groups were rendered diabetic and included untreated diabetic rats (D) and diabetic rats receiving quinapril at the dosages of 0.625 (DQ1), 2.5 (DQ2), 1.25 (DQ3), and 0.625 (DQ4) mg · kg\(^{-1}\) · day\(^{-1}\). Dosage-dependent effects of quinapril on systolic blood pressure and the glomerular filtration rate were observed. In contrast, control of P_{ab} in isolated glomeruli exposed to oncotic gradients, proteinuria, and glomerular and tubular hypertrophy was obtained with subhypotensive dosages (DQ3 and DQ4 groups) of the ACE inhibitor. In the in vitro study, quinapril reduced P_{ab} significantly in concentration ranges from 10\(^{-6}\) to 10\(^{-14}\) mol/l compared with results in control glomeruli. The effect on P_{ab} may have occurred by mechanisms different from kidney ACE inhibitor. These study results indicated that ACE inhibitor treatment prevents the early onset of the P_{ab} defect in experimental diabetes. This effect seemed to occur independently of systemic or glomerular hemodynamic changes and, at least partially, from kidney ACE inhibition. *Diabetes* 50:2619–2624, 2001

Microalbuminuria is an early marker of diabetic nephropathy (1), and its presence is indicative of an increased risk not only of overt nephropathy (2) but also of cardiovascular disease and death (3,4). The onset of microalbuminuria in diabetes is thought to result from increased glomerular passage of albumin and an eventual decrease in the protein reabsorptive capacity of the tubules (5).

Recently, we demonstrated that an albumin permeability (P_{ab}) defect exists in the earliest stages of streptozotocin (STZ)-induced type 1 diabetes by using the model of isolated nonperfused rat glomeruli, and we also showed that this alteration precedes the appearance of glomerular hyperfiltration and hypertrophy (6).

Treatment with ACE inhibitors has been shown to lower urinary protein excretion in experimental and human diabetes (7–10), as well as in other forms of renal disease (11–14). It is widely assumed that alterations in systemic and renal hemodynamics underlie the reported effect of ACE inhibitors in reducing proteinuria (15–17). Recently, it has been suggested that low dosages of ACE inhibitors may modify glomerular size selectivity in subjects with established diabetic nephropathy (18), thereby raising the possibility of a specific intrarenal effect of ACE inhibitors. However, it is still difficult to discriminate the role of hemodynamic and nonhemodynamic effects of ACE inhibitors in the prevention of diabetes-associated renal functional and structural modifications (19).

The main objective of the present study was to evaluate ex vivo the effects of treatment with hypotensive and subhypotensive dosages of the ACE inhibitor quinapril on the P_{ab} defect found in the early phases of experimental diabetes. To this end, we used an experimental procedure that excludes the presence of perfusion pressure as a variable in the analysis of permeability function. We simultaneously explored the possibility of using subhypotensive dosages of an ACE inhibitor to modulate the other functional and structural alterations that characterize the early phases of diabetes.

Although most of the relevant literature on the nephroprotective effect of ACE inhibitors in diabetes supports the view that inhibition of renal angiotensin II (ANG-II) generation by ACE inhibitors could prevent the renal hemodynamic (7) and structural changes (8) associated with...
diabetes, the extent to which inhibition of kidney ACE activity contributes to the nephroprotective effect of ACE inhibitors remains to be defined. Consequently, a further aim of this study was to correlate the inhibition of kidney ACE activity by different dosages of quinaprilat, the active metabolite of quinapril, with P\textsubscript{alb} in vitro in isolated glomeruli from diabetic rats.

**RESEARCH DESIGN AND METHODS**

**Study protocol**

*Ex vivo study*. Six groups of adult male Wistar rats (n = 10 in each group) with initial weights of 250–270 g were studied for 4 weeks. One group served as a nondiabetic control (C); the other five groups were rendered diabetic by intravenous injection of STZ (55 mg/kg, Sigma, St. Louis, MO) in citrate buffer. Control animals were injected with citrate buffer only. Only animals with blood glucose levels >15 mmol/l 2 days after the induction of diabetes were included as diabetic animals in the study.

Rats were housed in groups of three per cage in an air-conditioned, light-controlled environment and fed a normal diet containing ~24% protein by weight (Harlan Nossan Correzzana, Milan, Italy). All animals had unrestricted access to food and water. The diabetic animals were randomly allocated to one of five groups. The first diabetic group (D) received no specific therapy. The other groups were treated with quinapril (Parke-Davis, Ann Arbor, MI) at dosages of 0.025 (DQ2), 0.25 (DQ4), 1.25 (DQ5), and 0.625 (DQ6) mg · kg\textsuperscript{-1} · day\textsuperscript{-1} in drinking water. The diabetic rats received daily evening injections of 8 IU of ultralente insulin (Ultralente MC; Novo Nordisk, Copenhagen, Denmark) to maintain blood glucose at ~22–28 mmol/l and improve long-term survival.

At 2-week intervals, the systolic blood pressure (SBP) was measured in conscious, restrained, preheated rats by tail-cuff plethysmography (20). The rats were then placed in individual metabolic cages for 24 h, and their urine was collected for measurement of volume and total urinary protein. The day after the metabolic study, a venous blood sample from rats under ether anesthesia was also drawn and centrifuged; the serum was frozen for later analysis of glucose, creatinine, and ACE activity.

At the end of the study, the glomerular filtration rate (GFR) was evaluated by the determination of iopamidol (Iopamiro; Schering AG, Berlin, West Germany) clearance. After the rats were decapitated, their abdomens were opened and the kidneys were rapidly excised and weighed. P\textsubscript{alb} was determined by the determination of iopamidol (Iopamiro; Schering AG, Berlin, West Germany) clearance. The kidney was frozen in liquid nitrogen and stored at −80 °C for evaluation of glomerular and proximal tubular areas. The remaining 75% of the kidney was homogenized on ice in potassium phosphate buffer (pH 6.9 to 7.0) and the urine was collected for measurement of albumin and creatinine.

In the in vitro study, a venous blood sample from rats under ether anesthesia was also drawn and centrifuged; the serum was frozen for later analysis of glucose, creatinine, and ACE activity.

At the end of the study, the glomerular filtration rate (GFR) was evaluated by the determination of iopamidol (Iopamiro; Schering AG, Berlin, West Germany) clearance. After the rats were decapitated, their abdomens were opened and the kidneys were rapidly excised and weighed. P\textsubscript{alb} was determined in glomeruli isolated from the renal cortex of one of the two kidneys. Then 25% of the contralateral kidney was fixed in neutral formalin, processed through graded ethanol solutions, cleared in xylol, and embedded in paraffin for evaluation of glomerular and proximal tubular areas. The remaining 75% of the kidney was frozen in liquid nitrogen and stored at −80 °C for ACE activity measurement.

In the in vitro study, adult male Wistar rats were killed 4 weeks after induction of diabetes. In a first group of five animals, P\textsubscript{alb} was evaluated with or without 20 min of preincubation with different concentrations (10\textsuperscript{-10}, 10\textsuperscript{-9}, 10\textsuperscript{-8}, 10\textsuperscript{-7}, and 10\textsuperscript{-6} mol/l) of quinaprilat (Parke-Davis), the major active metabolite of quinapril. The second series of animals was used for evaluating the effect of the same concentrations of quinaprilat on kidney ACE activity. Concomitantly, the in vitro inhibitory potency of quinaprilat on kidney ACE activity was tested in a concentration range from 10\textsuperscript{-10} to 10\textsuperscript{-4} mol/l, considering that the quinaprilat IC\textsubscript{50} has been estimated to be 4.5 × 10\textsuperscript{-4} mol/l (21).

The procedures followed in this study were in accordance with institutional guidelines for experimental animal research.

**Isolation of glomeruli and measurement of P\textsubscript{alb}**. Glomeruli were isolated by standard sieving techniques in medium containing the following (in mmol/l): sodium chloride 115, potassium chloride 5, sodium acetate 10, dibasic sodium phosphate 1.2, sodium bicarbonate 25, magnesium sulfate 1.2, calcium chloride 1.0, and glucose 5.5. The pH was titrated to 7.4. The medium also contained 0.1 g/l bovine serum albumin (BSA) as an oncotic agent. The isolated glomeruli, which were free from capsules and arterioles, were then washed in 1 ml fresh medium; an aliquot of 0.1 ml was then incubated in 0.9 ml of medium for 10 min at 37°C. Using an in vitro study design, parallel incubations were run with the addition in the medium of quinaprilat at different concentrations (10\textsuperscript{-10} to 10\textsuperscript{-4} mol/l). The incubated glomeruli with or without the test agent were then transferred to a glass coverslip coated with poly-L-lysine and covered with fresh medium.

The rationale and methodology for the determination of P\textsubscript{alb} has been previously described in detail (22). In brief, each 10–16 glomeruli per animal were videotaped through an inverted microscope before and after a medium exchange to one containing 10 g/l BSA. The medium exchange created an oncotic gradient across the basement membrane, resulting in a glomerular volume change (ΔV = (V\textsubscript{water} − V\textsubscript{albumin})/V\textsubscript{albumin}), which was measured off-line by a video-based image analysis program (SigmaScan Pro; Jandel Scientific Software, Erkrath, Germany). The computer program determines the average radius of the glomerulus in two-dimensional space, and the volume is derived from the formula V = 4/3πr\textsuperscript{3} mm\textsuperscript{3}. The magnitude of ΔV was related to the albumin reflection coefficient, σ\textsubscript{alb}, by the following equation: (σ\textsubscript{alb,experimental} − σ\textsubscript{alb,control}) / σ\textsubscript{alb,control}. The σ\textsubscript{alb} of the control glomeruli was assumed to be equal to 1. P\textsubscript{alb} was defined as (1−σ\textsubscript{alb}) and described the movement of albumin subsequent to water flux. When σ\textsubscript{alb} is zero, albumin moves across the membrane with the same velocity as water and P\textsubscript{alb} is 1.0; conversely, when σ\textsubscript{alb} is 1.0, albumin cannot cross the membrane with water and P\textsubscript{alb} is zero. These assumptions are valid only when the response to an impermeable solute, such as high–molecular weight neutral dextran, is not affected by the experimental manipulations (23).

**Assessment of renal morphometry**. Two microribbon sections 3–5 μm thick from each kidney were stained with hematoxylin and eosin and examined by light microscopy. Each section was placed in a microscope and recorded by a color video camera; the image was then transferred via computer to a video-based image analysis program (MCID; Imaging Research, St. Catharines, Ontario, Canada). The areas of 100 consecutive glomerular tufts sectioned at the vascular pole and 100 proximal renal tubules were measured by two independent observers. Interobserver variability between measurements for all sections was <5%. The reported values of the glomerular and proximal tubular areas represent the mean of the measurements obtained by both examiners.

**Fluorometric assay of ACE**. Serum and kidney ACE activities were estimated by a modification of the method of Cushman and Cheung (24). On the day of assay for ACE activity, the frozen kidneys were weighed, placed in fresh test tubes, and homogenized on ice in potassium phosphate buffer (pH 8; 1.5 wt/vol) containing 0.3% Triton X-100 using an Ultra Turrax T-25 (13,500 rpm) for 10 s. After centrifugation (15 min at 15,000 rpm at 4°C), ACE was assayed in the supernatant. Plasma or tissue homogenates were diluted with 0.25 ml of the incubation mixture, composed of 0.1 mol/l phosphate buffer (pH 8.0), 0.05 mol/l NaCl, and 1.0 mmol/l His-Leu-Enkephalin. The experiments measuring the inhibitory potency of quinaprilat, diluted tissue preparations were mixed with 0.05 ml of serial dilution of the ACE inhibitor (from 10\textsuperscript{-6} to 10\textsuperscript{-11} mol/l). The enzymatic reactions were stopped after 15 min by the addition of 1.5 ml of 280 mmol/l NaOH. The liberated His-Leu was converted into a fluorescent product by adding, with rapid mixing, 0.1 ml of 1% orthophosphaldehyde in methanol. After 20 min, the reaction was terminated by adding 0.2 ml of 3N HCl. All tubes were centrifuged for 5 min at 2,000 rpm, and fluorescence was measured after 30 min at an excitation wavelength of 360 nm and an emission wavelength of 400 nm. All measurements were done in duplicate, and all ACE samples were run in parallel with millimolar EDTA, an ACE inhibitor, to correct for nonspecific fluorescence.

**Statistical analysis**. Data are reported as means ± SE. Comparisons among different rat groups over the study period were performed by analysis of variance (ANOVA) and a subsequent Scheffe’s test. Parameters within one group at different time points were compared by ANOVA for repeated measures. Linear regression analysis was used for testing two variable relationships. Statview 512 software for the Apple Macintosh computer was used. A value of P < 0.05 was required for significance.

**RESULTS**

**Weight and blood glucose control**. Data for body weight, blood glucose control, and urine volume are shown in Table 1. During the experimental period, diabetic animals gained less weight than did control rats. Blood glucose and urine volumes were greatly increased in the diabetic rats, as expected (P < 0.01). Antihypertensive therapy did not influence body weight at any of the
dosages used. Comparable values of serum glucose and urine volumes were maintained in all the diabetic groups throughout the study period.

SBP. The SBPs for the six groups are shown in Fig. 1. There was no significant difference in blood pressure between diabetic and nondiabetic rats. Quinapril decreased the SBP in a dosage-dependent manner in the four treated groups, but only the higher dosages of quinapril (groups DQ1 and DQ2) resulted in a statistically significant decrease in blood pressure ($P < 0.01$).

**Glomerular $P_{\text{alb}}$.** The $P_{\text{alb}}$ was increased in diabetic animals (Fig. 2). Treatment with the ACE inhibitor quinapril markedly reduced $P_{\text{alb}}$, independent of the dosages used. $P_{\text{alb}}$ was closely related to proteinuria ($r = 0.463$, $P = 0.002$), whereas no correlation was found between $P_{\text{alb}}$ and GFR. A positive but not statistically significant correlation between $P_{\text{alb}}$ and blood pressure was found.

Incubation of isolated glomeruli from diabetic rats with quinaprilat in concentrations ranging from $10^{-6}$ to $10^{-15}$ mol/l for 10 min at $37^\circ$C significantly reduced $P_{\text{alb}}$ as compared with results in control glomeruli (Fig. 3).

**Renal function and structure.** The induction of diabetes was associated with a significant increase in the GFR. In the DQ1 and DQ2 groups, after 4 weeks of treatment with 5 and 2.5 mg · kg$^{-1}$ · day$^{-1}$, respectively, the increase in GFR was completely prevented. In contrast, no effect on this parameter was seen after an equivalent period of treatment with 1.25 and 0.625 mg · kg$^{-1}$ · day$^{-1}$ of quinapril (DQ3 and DQ4 groups, respectively) (Table 2). The 24-h total urinary protein excretion was significantly increased in the untreated diabetic animals compared with the control and treated diabetic animals. The increase in proteinuria was attenuated by quinapril treatment, independent of the dosages used (Table 2). No relation was found between proteinuria and GFR.

Serum creatinine rose similarly in all the diabetic animals (Table 2).

In contrast to somatic growth, diabetes was associated with renal hypertrophy. The ratio of two-kidney weight to body weight was significantly higher in the diabetic rats than in the nondiabetic control rats ($P < 0.05$) (Table 2). The renal morphometric measurements showed an increase in glomerular and proximal tubular areas in the diabetic compared with the nondiabetic rats (Fig. 4). Quinapril treatment at subhypotensive dosages also prevented the hypertrophic response of glomerular and proximal tubular cells (Fig. 4).

**Plasma and kidney ACE activity.** No differences were

### Table 1

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<th>Parameters</th>
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<td>300 ± 7*</td>
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<td>72.6 ± 11.6*</td>
<td>60.6 ± 12†</td>
<td>71.1 ± 11.6*</td>
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Data are means ± SE. $C$, control rats; $D$, diabetic rats; $DQ1$–$DQ4$, diabetic rats treated with quinapril at 5 ($DQ1$), 2.5 ($DQ2$), 1.25 ($DQ3$), and 0.625 ($DQ4$) mg · kg$^{-1}$ · day$^{-1}$. *$P < 0.01$, †$P < 0.05$ vs. C group.

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**FIG. 1.** Sequential values for SBP for the six animals groups throughout the experimental period. $C$, control rats; $D$, diabetic rats; $DQ1$–$DQ4$, diabetic rats treated with quinapril at 5 ($DQ1$), 2.5 ($DQ2$), 1.25 ($DQ3$), and 0.625 ($DQ4$) mg · kg$^{-1}$ · day$^{-1}$. Data are means ± SE. *$P < 0.01$ vs. $C$, $D$, $DQ3$, and $DQ4$ groups.

**FIG. 2.** $P_{\text{alb}}$ determined in isolated glomeruli exposed to oncotic gradients during the course of the experiment. $C$, control rats; $D$, diabetic rats; $DQ1$–$DQ4$, diabetic rats treated with quinapril at 5 ($DQ1$), 2.5 ($DQ2$), 1.25 ($DQ3$), and 0.625 ($DQ4$) mg · kg$^{-1}$ · day$^{-1}$. Data are means ± SE. *$P < 0.01$ vs. control, $DQ1$, $DQ2$, $DQ3$, and $DQ4$ groups.
In our study, the administration of subhypotensive dosages of quinapril to diabetic rats prevented the $P_{\text{ab}}$ defect and proteinuria without affecting the glomerular hyperfiltration response to hyperglycemia. The use of isolated glomeruli, in contrast to studies of dextran-sieving profiles (25–28), permitted us to assess the capillary $P_{\text{ab}}$ without the influence of pressure and flow variables. The prevention of the $P_{\text{ab}}$ defect by low dosages of quinapril was associated with a significant inhibition of kidney ACE activity. Collectively, these data are consistent with a nonhemodynamic effect of ACE inhibitors on glomerular filtration barrier permeability properties. Considering that ANG-II has been implicated as a stimulus for matrix production from mesangial cells (29) and for remodeling of the glomerular basement membrane in diabetes (30), the favorable effect of ACE inhibition on $P_{\text{ab}}$ could be the consequence of long-term inhibition of ANG-II generation in the kidney. On the other hand, some of the favorable effects of ACE inhibition on $P_{\text{ab}}$ may be explained by actions other than those on tissue in the renin angiotensin system. Comparing the dosage-related effects of quinapril on kidney ACE activity with the effects on $P_{\text{ab}}$, it was evident that quinapril reduced $P_{\text{ab}}$ at dosages below the threshold for ACE inhibition. Although we cannot exclude

**DISCUSSION**

The principal finding of the present study was that treatment with the ACE inhibitor quinapril prevented the $P_{\text{ab}}$ defect when administered at subhypotensive dosages; a secondary finding was that significant improvement in the permeability defect occurred in vitro by incubating isolated glomeruli from diabetic rats with different dosages of quinapril. A direct effect of ACE inhibitors in improving glomerular size selective properties has been previously found in rat models of genetically determined, age-dependent glomerulosclerosis (25) and in humans with diabetic nephropathy (26) and other nondiabetic proteinuric renal diseases (27). In contrast, glomerular size selective dysfunction and proteinuria have not been shown to be ameliorated by ACE inhibition in type 2 diabetes, probably because the structural changes in type 2 diabetes are often advanced and diffuse, involving thickening of the basement membrane, broadening of the foot processes, and diffuse sclerotic changes (28).

In our study, the administration of subhypotensive dosages of quinapril to diabetic rats prevented the $P_{\text{ab}}$ defect and proteinuria without affecting the glomerular hyperfiltration response to hyperglycemia.
the possibility that the dissociation between the effects of quinaprilat on inhibition of ACE activity and the amelioration of permeability of the glomerular barrier is a consequence of the different tissue preparation used in the in vitro experiments, the favorable effect of quinaprilat on the glomerular barrier may have involved mechanisms different from the inhibition of "classic" ACE. ACE inhibitors may block kinases other than ACE, including enzymes that are related to or are isomers of ACE (31) or aminopeptidase P, or those that may be related to endopeptidase 24.15 (32) or matrix metalloproteinases (33). The inhibition of non-ACE kinases by quinaprilat may be an alternative or synergistic mechanism by which ACE inhibitors prevent or reduce the glomerular permeability defect.

In the present study, we recapitulated many previous experiments that showed that hyperfiltration, proteinuria, and renal hypertrophy were prevented by ACE inhibitor treatment (7,8,15). It is interesting to note, however, that although the effects on GFR and blood pressure clearly depended on the dosage, the prevention of increased urinary protein excretion and morphometric changes of glomerular and tubular structure was also evident with subhypotensive dosages of ACE inhibitor. This would indicate that a reduction in systemic blood pressure or in the glomerular filtration rate is not a prerequisite for the antiproteinuric and antirenal growth effect of ACE inhibitors in the early phases of diabetic nephropathy. The discrepancy observed with the subhypotensive dosages of quinapril between reduction of proteinuria and the absence of significant effects on glomerular hyperfiltration is consistent with the notion that the major contributor to increased glomerular filtration of albumin is not hyperfiltration but an increase in macromolecular permeability at the glomerular filtration barrier (5,34). Accordingly, in our

**FIG. 4.** Glomerular (A) and tubular (B) areas among groups 4 weeks after induction of diabetes. C, control rats; D, diabetic rats; DQ1–DQ4, diabetic rats treated with quinapril at 5 (DQ1), 2.5 (DQ2), 1.25 (DQ3), and 0.625 (DQ4) mg·kg⁻¹·day⁻¹. Data are means ± SE. *P < 0.01 vs. C, DQ1, DQ2, DQ3, and DQ4 groups.

**FIG. 5.** In vitro inhibition of converting enzyme in rat kidney by quinaprilat.

**FIG. 6.** Comparison of the in vitro effect of increasing concentrations of quinaprilat (10⁻⁶, 10⁻⁷, 10⁻¹⁰, 10⁻¹², 10⁻¹⁴, and 10⁻¹⁵) on glomerular P₁₀₀ and kidney ACE activity in diabetic (D) rats. Reduction of P₁₀₀ and inhibition of kidney ACE activity are expressed as percentages of the values in the untreated animals.
study, a positive correlation was found between proteinuria and $P_{\text{ab}}$ whereas proteinuria and the GFR were not correlated.

The capacity of ACE inhibitors to prevent renal growth is well known; however, there has been no previous evidence of a beneficial effect of subhypotensive dosages of ACE inhibitors on the diabetes-related renal hypertrophic response. The reduction of proteinuria by ACE inhibitors may attenuate the growth response of tubular cells (35). However, the direct evidence in this study for kidney-converting-enzyme inhibition after chronic treatment with quinapril supports the notion that a specific effect of renal ACE inhibition may be important for preventing renal structural modifications in the course of diabetes.

In conclusion, our data document the capacity of ACE inhibitors to prevent the $P_{\text{ab}}$ defect existing very early in the course of incipient diabetic nephropathy in STZ-administered rats. This effect seems to have been independent of systemic and glomerular hemodynamic changes. Our results also indicate a positive effect of subhypotensive dosages of quinapril on renal hypertrophy. Finally, we have provided evidence that inhibition of kidney ACE activity may not be the only determinant of the improvement in $P_{\text{ab}}$, thus giving new insights into the mechanisms responsible for the nephroprotective effect of ACE inhibitors in the course of diabetic nephropathy. However, the confirmation of this concept would ultimately require similar designed studies with ANG-II receptor antagonists.

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REFERENCES