Salt Blocks the Renal Benefits of Ramipril in Diabetic Hypertensive Rats

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To establish if the benefit of angiotensin converting enzyme inhibitor therapy in retarding progressive diabetic renal injury is due to a specific intrarenal effect or the systemic hypotensive effect, we studied the effect of long-term ramipril treatment on blood pressure, glomerular filtration rate, and urinary protein excretion in streptozotocin-diabetic spontaneously hypertensive rats. The hypotensive effect of ramipril was prevented by a high salt diet, which did not alter the degree of renal angiotensin converting enzyme inhibition. Three weeks after uninephrectomy and induction of diabetes, rats were allocated to three groups. Groups 1 and 2 were given 1% NaCl, whereas group 3 was given water as drinking solution. One week later, groups 2 and 3 received 0.4 mg/kg/day ramipril in their drinking solution, which was continued over a 2-month period. Ramipril produced a blood pressure fall only in water-drinking rats (group 3) despite a similar reduction in plasma and renal angiotensin converting enzyme activity in groups 2 and 3. Salt-loaded rats had a progressive increase in urinary protein excretion over the duration of study. Ramipril treatment prevented an increase in protein excretion only in animals given water and with a reduced systolic blood pressure. Glomerular filtration rate was similar in all three groups. Ramipril treatment improved animal survival independently of a reduction in blood pressure or an effect on proteinuria. Although it is possible that angiotensin converting enzyme inhibitors have specific intrarenal effects reducing progression of diabetic proteinuria, concomitant control of systemic blood pressure appears to be necessary to demonstrate a benefit. (Hypertension 1991;17:497–503)

Uncontrolled systemic hypertension is a well-known cause of progressive renal injury in both humans and experimental animals.1

Hypertension is commonly associated with diabetic renal disease,1–3 and it has been shown to accelerate the rate of development of diabetic nephropathy both in humans4 and in streptozotocin-diabetic rats.5 Furthermore, clinical studies have suggested that antihypertensive therapy can reduce albuminuria and the progression of diabetic renal disease.6,7

More recently angiotensin converting enzyme (ACE) inhibitors have been shown to be as effective, if not superior to, conventional hypotensive agents in retarding the progression of renal injury in hypertensive8,9 and normotensive10,11 diabetic patients, in rats with streptozotocin diabetes,12,13 or in other renal disorders.14,15 However, it is difficult to determine from these studies if the beneficial effect of ACE inhibitors was due to a specific intrarenal effect of ACE inhibition or due to their systemic hypotensive effect.

To evaluate this question further, we examined the effect of long-term administration of the ACE inhibitor ramipril on blood pressure, glomerular filtration rate, and proteinuria in streptozotocin diabetic uninephrectomized spontaneously hypertensive rats (SHR). To prevent the hypotensive effect of ACE inhibition, the rats were maintained on a high salt diet, which is known to oppose the blood pressure-lowering action of ACE inhibitors.16,17

Methods

Study Protocol

Three groups of male Okamoto SHR bred at the Biological Research Laboratories, Austin Hospital, Heidelberg, Australia, were studied for a period of 12 weeks before they were killed. Rats 8–10 weeks old and weighing between 250 and 280 g were uninephrectomized under ether anesthesia and were made diabetic by intravenous injection of 45 mg/kg streptozotocin. Rats were caged in groups of three and fed a diet (Clark King & Co., Melbourne, Victoria, Australia) containing 20% protein, 0.8% potassium, and 0.3% sodium by weight.
Four days later, after diabetes was confirmed by an elevated blood glucose level (more than 15 mmol/l), treatment was commenced with 4 units of insulin zinc suspension, crystalline (Ultralente MC, Commonwealth Serum Laboratories, Melbourne, Australia, and Novo Industri A/S, Copenhagen, Denmark) 5 times/wk to reduce the severity of diabetes and improve long-term survival. This maintained blood sugars of approximately 30 mmol/l.

Three weeks after uninephrectomy and induction of diabetes, rats were randomly allocated to one of three groups. Two groups (groups 1 and 2) were given 1% NaCl as a drinking solution instead of water, which was given to group 3. One week later, therapy with 0.4 mg/kg/day ramipril was started in groups 2 and 3. The drug was added to the drinking solution, and therapy was continued throughout the next 8 weeks.

ACE activity was measured in serum obtained by tail bleed before and after 8 weeks of treatment. At 2-week intervals, systolic blood pressure was measured in conscious, restrained preheated rats by tail-cuff plethysmography. Rats were then placed in individual metabolic cages for 24 hours, and the urine was collected for measurement of urinary total protein, albumin, sodium, and potassium excretion. The day after the metabolic study, a venous blood sample was also taken and centrifuged; the serum was frozen for later analysis of glucose and creatinine. After 1 and 2 months of treatment, glomerular filtration rate was measured.

**Assay Methods**

Serum glucose was measured by a glucose oxidase technique. Serum and urinary creatinine were determined by an autoanalyzer technique (Beckman Instruments Inc., Fullerton, Calif.). Urinary sodium and potassium concentrations were determined by flame photometry. Proteinuria was assessed by measurement of 24-hour urinary total protein, albumin, sodium, and potassium excretion. Total urinary protein was assayed using the Coomassie dye binding technique. Urinary albumin excretion was measured by a coated tube radioimmunoassay with an interassay coefficient of variation of 10.4% and a lower detection limit of 5 μg/ml.

Serum ACE was measured by an enzyme kinetic method with hippuryl-L-histidyl-L-leucine as synthetic substrate. Renal ACE was measured by radioligand binding as described in detail previously. Ligand binding in kidneys obtained when the rats were killed at the end of the study period was expressed as a percentage of binding obtained in five nephrectomy specimens obtained before randomization.

**Renal Clearance Studies**

Glomerular filtration rate was determined using the isotope technetium-99m diethyleneetriaminepentaacetic acid (TcDTPA). TcDTPA was injected via a tail vein, and blood sampling was performed at 43 minutes. This method, a noninvasive single injection technique, allows glomerular filtration rate to be measured repeatedly in the same animal without chronic vascular cannulation. This method also avoids the problem of overestimation of glomerular filtration rate by creatinine clearance, which occurs in diabetes because of the presence of pseudocreatinines.

**Statistical Analysis**

Urinary albumin and protein excretion was analyzed after logarithmic transformation. Geometric means are shown for urinary albumin and protein excretion. The χ² test was used to assess mortality rates. Comparisons among different groups of rats over the study period were performed by analysis of variance with repeated measures using the CLR ANOVA program (Clear Lake Research, Houston, Tex.) for the Apple Macintosh computer. Where multiple comparisons were performed between groups, Tukey's method was used. Data are reported as the mean±SEM. A value of p<0.05 was required for significance.

The study was approved by the local ethics committee and conformed to the National Health and Medical Research Council of Australia guidelines for the use of animals in medical research.

**Results**

In the first 3 weeks after uninephrectomy and diabetes induction, 25 of the original 66 rats died. One of the rats of group 3 was killed because a submandibular abscess developed and for this reason was excluded from the survival analysis. Over the next 8 weeks, 14 of 18 rats in group 1, 4 of 13 rats in group 2, and 2 of 10 rats in group 3 died (Figure 1). The survival of the animals in the groups given ramipril was significantly improved (p<0.01).

Systolic blood pressure for the three groups is shown in Figure 2A. There was no significant differ-
ence in the basal values of systolic blood pressure among the groups. The increase in systolic blood pressure after uninephrectomy and induction of diabetes was also similar in all three groups. In rats receiving saline (group 1) in the drinking fluid, blood pressure continued to increase over the study period \((p<0.01)\), and the antihypertensive response to 0.4 mg/kg/day ramipril was completely abolished (group 2) \((p<0.01)\). In the group of rats fed a normal sodium diet (group 3), there was a significant reduction \((p<0.02)\) in blood pressure when the ACE inhibitor was added to drinking water, and this reduction was maintained throughout the study.

The differences in blood pressure between the group receiving water and ramipril (group 3) and that receiving saline and ramipril (group 2) to drink were present \((p<0.001)\) despite a similar reduction in the ACE activity in both plasma and the kidney (see Table 1).

Assessment of 24-hour urinary total protein and urinary albumin excretion confirmed that all three groups had similar levels of proteinuria (Table 1) and albuminuria (Figure 2B) at the start of the study. In the salt-loaded rats, there was a progressive increase in urinary protein and albumin excretion over the study period \((p<0.0001)\). Ramipril treatment prevented the increase in urinary protein and albumin excretion only in the water-drinking group of rats \((p<0.01)\) in which a concomitant reduction in systolic blood pressure was also seen.

There was no significant difference in glomerular filtration rate, as estimated by \(^{99m}\)TcDTPA clearance (ml/min/100 g rat), at either of the two time points among the three groups (Figure 3).

Serum creatinine rose similarly in all groups at each 2-week interval after uninephrectomy and diabetes induction (Table 1). There were no clinical signs of uremia in any of the rats during the studied, and there was no evidence of an excessive rise in serum creatinine, measured at 2-week intervals, in animals that died during the study period.

Blood glucose (mmol/l) was similar in each group before induction of diabetes, after induction of diabetes, and before ramipril treatment, and in all three groups of rats during the next 8 weeks of the study period (Table 1). All the rats had an osmotic diuresis. The urine volume and sodium excretion was higher in the two groups of rats (group 1 and 2) given 1% NaCl to drink \((p<0.001)\), and there was no difference in urinary volume or sodium excretion between the two NaCl-drinking groups (Figure 4).

At the end of the study period, kidney weight (both absolute weight and the kidney weight/body weight ratio) of surviving rats was similar in all three groups. Kidney weights (g) were: group 1, 1.82±0.10; group 2, 1.77±0.12; group 3, 1.75±0.08.

There was no difference in weight (g) or weight gain, as evaluated at the end of the study, among the three different groups (group 1, 263±12; group 2, 267±10; group 3, 252±10).

Discussion

The model of diabetes mellitus used in this study, combining genetic hypertension with streptozotocin diabetes, has been shown to be an excellent model.
for evaluating the importance of hypotension and the role of hypotensive agents in diabetic renal disease.\textsuperscript{28} The development of diabetic renal injury was accelerated by uninephrectomy, which has been shown to increase glomerular capillary hydraulic pressure and plasma flow rate in nondiabetic rats.\textsuperscript{29}

This study confirmed that in SHR a high salt intake prevents the hypotensive effect of chronic ACE inhibitor therapy.\textsuperscript{16,17} Treatment with ramipril produced a fall in systemic systolic blood pressure only when administered to rats receiving a normal salt intake.

The behavior of proteinuria paralleled that of blood pressure. Proteinuria progressively increased in diabetic rats over the study period, consistent with the development of diabetic nephropathy. Treatment with ramipril was able to reduce protein and albumin excretion only when accompanied by control of systolic blood pressure.

It is well-known that hypotensive therapy has an important role in preventing the progression of experimental nephropathy.\textsuperscript{6,7} Recent reports have suggested that treatment with ACE inhibitors may have a specific therapeutic advantage over other forms of systemic blood pressure control in slowing the progression of experimental renal failure in rats. In the renal ablation model of renal failure, ACE inhibitors have been shown to preserve glomerular filtration rate, lessen proteinuria, diminish the degree of focal glomerular sclerosis, and prolong life.\textsuperscript{12,13,28,29} Also, in rats with autologous immune complex nephritis (Heymann nephritis), both enalapril and captopril prevented increased urinary albumin excretion.\textsuperscript{31} Similarly, enalapril was able to reduce urinary protein excretion in a nephrotoxic serum nephritis model of renal failure.\textsuperscript{32} In experimental diabetic nephropathy, ACE inhibitors have been shown to prevent the development of albuminuria and glomerulosclerosis in normotensive streptozotocin-diabetic rats.\textsuperscript{12,13} An advantage of ACE inhibitors over verapamil has been reported in uninephrectomized streptozotocin-diabetic rats.\textsuperscript{13} A recent report has suggested that ACE inhibition not only reduces proteinuria in experimental diabetes but also retards glomerular basement membrane thickening in normotensive and hypertensive diabetic rats.\textsuperscript{34} However,
the advantage of ACE inhibition was not confirmed in a preliminary report in which the effect of enalapril was similar to that of a combination of hydralazine and metoprolol in hypertensive diabetic rats. In clinical studies, the administration of ACE inhibitors to both hypertensive and normotensive diabetic patients with incipient nephropathy and microalbuminuria was effective in reducing proteinuria. However, none of these studies had an appropriate control group of patients treated with alternate antihypertensive therapy. If one compares the effects of ACE inhibition with conventional agents, as reported by Parving et al., both antihypertensive regimens were associated with a similar degree of reduction in urinary albumin excretion and rate of decline in glomerular filtration rate. Recently, a study of a small group of normotensive diabetics demonstrated an advantage of captopril over nifedipine treatment on urinary excretion of albumin. In contrast, a recent multicenter study of microalbuminuria in patients with type I and type II diabetes mellitus showed that both perindopril and nifedipine, which reduced blood pressure to similar degrees, were equivalent in efficacy in reducing microalbuminuria.

The mechanism of chronic action of ACE inhibitors is unclear. Angiotensin II mainly constricts the efferent arteriole, thus contributing to the maintenance of glomerular capillary pressure and glomerular filtration. Removal of angiotensin II leads to a reduction of intraglomerular pressure. However, micropuncture studies have shown that lowering the blood pressure to normotensive levels in diabetic SHR afforded protection against the development of early diabetic glomerulopathy independently of a reduction in glomerular hydraulic pressure. Angiotensin II has many other actions within the kidney, including effects on mesangial cell contraction and macromolecular uptake through the mesangium, tubular sodium reabsorption, and vasa recta blood flow, all of which might modulate the progression of intrarenal pathology in renal failure. Renal ACE is not influenced by changes in salt balance and is effectively inhibited by ACE inhibitors.

Our data does not provide information concerning the mechanism of antiproteinuric effect of ACE inhibition. The high salt diet prevented the hypotensive and antiproteinuric effect of ACE inhibition. This observation could suggest that the mechanism by which ACE inhibitors bring about renoprotection is mainly due to a lowering of systemic blood pressure, although clearly the blood pressure achieved in this study was elevated relative to nonhypertensive strains of rats (—120—140 mm Hg). Our study does not, however, exclude an effect of ramipril on glomerular capillary pressure or capillary permselectivity.

Changes in salt status have been shown not to affect plasma ACE activity in humans or plasma and tissue ACE activity in rats. Although theoretically one could expect maximal efficacy of ACE inhibitors in states of activation of the renin-angiotensin system, it is well-known that ACE inhibitors are also effective in low renin essential hypertension and in
situations where plasma renin activity is low, such as in the renal failure of the remnant kidney model\textsuperscript{15} or in experimental diabetes.\textsuperscript{12}

Dietary sodium intake has been reported to modulate renal growth and glomerular volume after reduction in renal mass in the rat with a high salt diet potentially moderating renal injury.\textsuperscript{45} In our present study, there was no apparent difference in renal weight among the three groups. Because of the experimental design, the present study does not allow a dissection of the interaction of salt status and ACE inhibitor drug treatment on renal growth.

In the present study, ramipril treatment improved animal survival independently of either a reduction in blood pressure or the degree of hyperglycemia. Although it is attractive to attribute deaths to metabolic complications of diabetes, this did not appear to be the case. Similar improvement in animal survival independent of blood pressure effects has been demonstrated in a group of nondiabetic, salt-loaded, stroke-prone SHR treated with enalapril.\textsuperscript{17} Other studies have shown that treatment with ACE inhibitors reduces blood pressure and prolongs life in experimental models of renal failure.\textsuperscript{13,15} Long-term intracerebral treatment with captopril can reduce the salt appetite in SHR.\textsuperscript{46} In the present study, it is unlikely that the benefit of ramipril treatment was connected with a reduction of salt intake. Blood pressure was persistently elevated and sodium excretion was similar in the groups given a high salt diet (Figures 2 and 4). The mechanism of improved survival in the enalapril-treated groups remains to be determined.

The present study demonstrated that ramipril treatment reduced urinary protein excretion only when blood pressure was lowered, whereas animal survival was improved independently of the blood pressure reduction. Without excluding a specific intrarenal effect, it is likely that the renal protective effect of ACE inhibitors is best achieved with concomitant reduction in blood pressure, whereas animal survival seems to occur by a mechanism other than blood pressure reduction.

**References**

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