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# THE INFLUENCE OF ENALAPRIL OR SPIRONOLACTONE ON EXPERIMENTAL CYCLOSPORIN NEPHROTOXICITY

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**Abstract**—Adult Sprague–Dawley rats treated daily for 14 days with 50 mg/kg cyclosporin A (CsA) exhibited nephrotoxicity, characterized by reduced glomerular filtration rate, decreased urinary sodium and potassium flow, tubular enzymuria and proximal tubular structural damage. Elevations in plasma renin activity (PRA) were observed on day 4, but returned to normal within 7 days. Co-treatment of animals for the 14 day period with enalapril (8 mg/kg/day), a potent inhibitor of angiotensin converting enzyme (ACE), or spironolactone (25 mg/kg/day), the distal tubular antagonist of aldosterone, reduced the nephrotoxicity, although PRA remained elevated. Neither enalapril nor spironolactone affected circulating CsA levels. These data suggest that the action of aldosterone on the distal tubule may be important in the pathogenesis of CsA nephrotoxicity.

Cyclosporin A (CsA) has become widely accepted as the agent of choice for the control of organ allograft rejection. Nephrotoxicity, however, is a common and often worrying side effect of the drug which may necessitate its withdrawal in some patients [1]. This property of CsA has, to date, restricted its evaluation as an immunotherapeutic agent in areas of clinical medicine other than transplantation. In both animals and man, CsA nephrotoxicity is characterized by impairment of glomerular filtration, enzymuria and structural damage to renal tubular cells [2–4]; glomerular capillary and arteriolar thromboses have also been ascribed to CsA [5, 6]. These changes appear to be reversible upon drug curtailment or withdrawal [4, 7, 8].

Several hypotheses have been propounded to explain the pathogenesis of CsA nephrotoxicity. Suggested underlying mechanisms include (i) a primary glomerular event [9]; (ii) a primary tubular lesion [10, 11], reducing glomerular filtration by increased back pressure along the nephron or via feedback activation of the renin–angiotensin–aldosterone system (RAAS) [12, 13]; (iii) alternative involvement of the RAAS because of reduced production of angiotensin II antagonists (prostacyclin and prostaglandin E), leading to hypertension and vascular lesions [6, 14].

CsA nephrotoxicity has been associated in man with hypertension [15], a recognized side effect of the drug, especially in cardiac allograft recipients [16]. In experimental animals, spontaneously hypertensive rats are particularly susceptible to CsA nephrotoxicity compared with normotensive counterparts [13]. Recent studies have shown that acute CsA nephrotoxicity in the rat is associated with elevated plasma renin activity (PRA) [13, 17–21].

In order to elucidate further the role of the RAAS in CsA nephrotoxicity, the present study examines the effect of angiotensin converting enzyme (ACE) inhibition and aldosterone blockade in animals receiving a known nephrotoxic dose of the immune suppressant.

## MATERIALS AND METHODS

*Animals*. Adult male Sprague–Dawley rats (mean weight 350 g), obtained from the University Animal Department, Foresterhill, Aberdeen, were used throughout. Food (Oxoid Pasteurised Breeding Diet) containing 0.23% w/w NaCl with Na:K of approx. 3:1 and tap water were freely available.

*Drugs*. Cyclosporin (CsA; OL-27-400), obtained in powder form from Sandoz Ltd., Basle, Switzerland, was initially dissolved in absolute ethanol at ambient temperature. Olive oil (Boots PLC Ltd., Nottingham, U.K.) was added to give a solution of 10% ethanol in olive oil (50 mg CsA/ml). CsA or its vehicle (ethanol/olive oil) was administered to the conscious animal, once daily, by oral gavage (0.3–0.4 ml), using a 4 FG intravenous cannula (Portex Ltd., Hythe, Kent, U.K.). Enalapril (MK 421: Merck, Sharpe & Dohme, Hoddesdon, Herts, U.K.) or spironolactone, as potassium canrenoate (Spiroctan-M injection, MCP Pharmaceuticals Ltd., Livingston, West Lothian, U.K.) was administered by a single daily intraperitoneal injection (0.3–0.55 ml).

*Protocol*. Groups of 6 rats received CsA (50 mg/kg) alone or in combination with either enalapril (8 mg/kg) or spironolactone (25 mg/kg) over 14 days. Animals receiving CsA vehicle, enalapril or spironolactone alone were also investigated. Samples of blood and urine were obtained immediately pretreatment and at the same time (early morning) prior to drug administration on days 4, 7 and 14. On day 14, the animals were killed by terminal ether anaesthesia and the kidneys removed for histological examination.

*Blood and urine collection*. Blood was obtained by tail clipping under light ether anaesthesia. It was placed in either cooled (4°) tubes (1.25 ml) containing K.EDTA as anticoagulant or plain tubes (1.0 ml) at room temperature. Plasma was obtained from the anticoagulated blood by centrifugation at 4000 r.p.m. for 10 min at 4° and stored at −20° until

required. The blood collected in plain tubes was allowed to clot for 1 hr at room temperature and the serum expressed by centrifugation stored at −20° until required.

Urine free of faecal contamination was obtained from rats kept in metabolic cages overnight (16 hr). Prior to the experiment, each animal was placed in a metabolic cage for 18 hr periods on three consecutive days. This allowed the animals to adapt to the new environment to which they were exposed during subsequent urine collections periods. A preliminary study had demonstrated that this period of conditioning reduced stress-related changes in renal function, enzymuria and PRA.

*Biochemical investigations.* Levels of urea, creatinine, sodium and potassium in samples of serum and urine were measured using an Astra Discrete Analyser (Beckman-RIIC Ltd., Glenrothes, Fife, U.K.). Clearance rates of both urea and creatinine were derived using the following equation:

$$\text{Clearance rate } (\text{ml. hr}^{-1}) = \frac{\text{Urine concentration}}{\text{Serum concentration}} \times \text{Urine flow rate } (\text{ml. hr}^{-1})$$

For determination of PRA, 500 μl of each plasma sample was mixed with 5 μl phenylmethylsulphonylfluoride and 50 μl maleate (45 mM; pH 6.0) at 4°. Following incubation of 250 μl of the buffer mixture for 90 min at 37°, angiotensin I levels were assayed using a Clinical Assays Coat radioimmunoassay kit. PRA was expressed as ng/ml/hr of generated angiotensin I. Urine activities of *N*-acetyl-β-D-glucosaminidase (NAG), determined by the method of Whiting *et al.* [22], were expressed as IU/hr/mmol urine creatinine, a measure independent of urine flow rate.

*Radioimmunoassay of cyclosporin.* Trough cyclosporin concentrations in serum samples obtained 20 hr after the last treatment were estimated using radioimmunoassay kits supplied by Sandoz Ltd., Basle, Switzerland. The assay, which was performed following the supplier's instructions, did not distinguish between the parent CsA molecule and certain of its metabolites.

*Light microscopy.* Kidneys were fixed in 10% neutral buffered formalin and processed to paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin and examined without knowledge of the treatment. The proportion of damaged tubular cell profiles was assessed using the following scoring system: (0), none; (1), <10%; (2), >10 < 50%; (3), >50%.

*Statistics.* Results were compared using analysis of variance followed by the appropriate use of Student's *t*-test for dependent or independent samples. P values <0.05 were considered significant.

## RESULTS

### *General observations*

Over the experimental period, animals treated with either enalapril or spironolactone alone demonstrated significant weight gain (19 and 28% respectively; $P < 0.001$) which did not differ significantly from controls; the weight of those treated with both CsA and enalapril remained steady, whilst significant decreases of 18% ($P < 0.05$) and 8% ($P < 0.01$) respectively, were noted in animals treated with either CsA alone or with CsA in combination with spironolactone. Daily food, salt and water intakes were similar in all experimental groups (26 ± 5 g, 1.35 ± 0.45 mmol and 65 ± 13 ml, respectively) over the experimental period, except on day 14, when a 50% decrease in water intake was observed in animals receiving CsA alone. Diarrhoea of similar severity was observed in animals of each treatment group.

### *Glomerular filtration rate (GFR) and enzymuria*

Groups of animals receiving the vehicle, enalapril or spironolactone alone over a 2-week experimental period demonstrated no significant changes in renal function (data not shown). The influence of CsA on renal function and enzymuria, when administered either alone or in combination with enalapril or spironolactone is shown in Table 1. Differences in pre-treatment (day 0) clearance values between groups were not statistically significant. In rats receiving only CsA, significant elevation in urinary NAG activity was detected by day 4, whereas significant impairment of glomerular filtration was not evident until day 7. A similar degree of renal dysfunction was observed at 14 compared with 7 days, but enzymuria, which was most marked on day 7 (fourfold elevation above normal), had decreased towards pretreatment values by day 14.

Similar, but less marked changes in renal function were observed in the CsA groups co-treated with enalapril or spironolactone. In the latter instance, the diuretic significantly reduced the extent of CsA-induced GFR impairment. With respect to urinary NAG levels, however, both the ACE inhibitor and the aldosterone antagonist significantly reduced the degree of tubulotoxicity evident by 7 and 14 days.

### *Sodium and potassium excretion*

Treatment with either enalapril or spironolactone alone had no effect on either sodium or potassium excretion, while a 3–4-fold reduction was noted over the first 7 days of the study in animals receiving CsA alone (Table 2), with no further significant change being observed at day 14. Animals co-treated with enalapril exhibited no significant change in sodium flow rate, whilst potassium excretion was significantly diminished on days 4 and 7. Excretion rates were also significantly decreased over the first 7 days in animals co-treated with spironolactone. However, in this latter group on day 14, urinary sodium and potassium flow rates were significantly increased compared to day 7 values ($P < 0.05$) and in the case of sodium flow, to treatment with CsA alone ($P < 0.01$).

Treatment with enalapril or spironolactone alone produced a progressive, significant increase in PRA (3.5-fold and 2-fold, respectively) over the experimental period (Table 3), whilst animals receiving CsA alone showed only a transient elevation in PRA (3.5-fold) on day 4. Co-administration of CsA with either enalapril or spironolactone resulted, from day 4 onwards, in a 3–4-fold elevation in PRA which was maintained until day 14.

Table 1. The effect of CsA alone or in combination with enalapril or spironolactone on renal function

| Treatment | Serum urea (mmol/l) | Urea Clearance (ml/hr/kg) | Serum creatinine (μmol/l) | Creatinine clearance (ml/hr/kg) | Urinary NAG (IU/mmol) |
|---|---|---|---|---|---|
| CsA alone | | | | | |
| Day 0 | 6.3 ± 1.2 | 247 ± 62 | 49 ± 7 | 283 ± 29 | 66 ± 16 |
| 4 | 6.6 ± 1.3 | 239 ± 59 | 47 ± 5 | 279 ± 33 | 148 ± 52* |
| 7 | 9.4 ± 1.8† | 116 ± 67‡ | 65 ± 10 | 173 ± 64† | 281 ± 68‡ |
| 14 | 12.3 ± 3.3† | 140 ± 13‡ | 75 ± 5† | 180 ± 40* | 131 ± 31‡ |
| CsA + enalapril | | | | | |
| Day 0 | 5.9 ± 1.0 | 348 ± 115 | 41 ± 4[a] | 348 ± 90 | 60 ± 8 |
| 4 | 6.9 ± 0.8 | 220 ± 96† | 49 ± 8 | 236 ± 58† | 117 ± 30‡ |
| 7 | 7.7 ± 0.9† | 188 ± 68† | 50 ± 5† | 211 ± 75† | 141 ± 36‡[c] |
| 14 | 9.3 ± 0.5* | 198 ± 42† | 53 ± 4†[c] | 244 ± 49† | 78 ± 27[a] |
| CsA + spironolactone | | | | | |
| Day 0 | 6.4 ± 0.6 | 432 ± 110 | 50 ± 3 | 298 ± 58 | 65 ± 14 |
| 4 | 6.8 ± 1.1 | 209 ± 43 | 50 ± 5 | 231 ± 65 | 113 ± 35† |
| 7 | 10.7 ± 2.1† | 144 ± 47† | 58 ± 13 | 229 ± 66 | 145 ± 42*[c] |
| 14 | 9.2 ± 2.4 | 224 ± 88†[a] | 56 ± 2[c] | 261 ± 31[b] | 70 ± 22[a] |

Results are expressed as the mean ± 1 SD and are compared to their corresponding pretreatment values by Student's *t*-test for paired samples: * $P < 0.01$; † $P < 0.05$; ‡$P < 0.001$. Corresponding results (including day 0 values) for animals treated with CsA alone or in combination with either enalapril or spironolactone were compared using Student's *t*-test for unpaired samples: [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

### *Morphological observations*

Histological examination of kidneys in the CsA-treated group at 14 days revealed characteristic, proximal straight tubular cell vacuolation. Co-administration of either enalapril or spironolactone reduced the number of tubular profiles affected (Table 4).

Table 2. Effect of CsA alone or in combination with enalapril or spironolactone on urinary sodium and potassium excretion

| Treatment | $Na^+$ flow (μmol/hr) | $K^+$ flow (μmol/hr) |
|---|---|---|
| CsA alone | | |
| Day 0 | 72 ± 21 | 240 ± 14 |
| 4 | 38 ± 13‡ | 141 ± 34† |
| 7 | 20 ± 13‡ | 83 ± 48* |
| 14 | 17 ± 3‡ | 152 ± 48† |
| CsA + enalapril | | |
| Day 0 | 54 ± 22 | 176 ± 36 |
| 4 | 47 ± 11 | 102 ± 25* |
| 7 | 35 ± 20 | 96 ± 45* |
| 14 | 46 ± 19[a] | 134 ± 27 |
| CsA + spironolactone | | |
| Day 0 | 68 ± 17 | 201 ± 42 |
| 4 | 31 ± 13† | 119 ± 39* |
| 7 | 27 ± 20† | 111 ± 32* |
| 14 | 49 ± 17†[a] | 175 ± 33 |

Results are expressed as the mean ± 1 SD and are compared to their corresponding pretreatment values by Student's *t*-test for paired samples: * $P < 0.01$; † $P < 0.05$; ‡ $P < 0.001$. Corresponding results (including day 0 values) for animals treated with CsA alone or in combination with either enalapril or spironolactone were compared using Student's *t*-test for unpaired samples: [a] $P < 0.01$.

### *Cyclosporin levels*

Trough serum cyclosporin levels are shown in Table 5. Similar values were obtained in all groups receiving CsA; neither enalapril nor spironolactone significantly affected drug concentrations.

## DISCUSSION

By demonstrating that co-treatment with an ACE-inhibitor or an aldosterone antagonist can reduce the well-known nephrotoxic effects of CsA, viz. impaired GFR and proximal tubular damage, this study has shown that the RAAS contributes to this common side effect of the immunosuppressant. Furthermore, the reduced electrolyte excretion rate in the presence of consistent food intake, was partially reversed by co-treatment with either spironolactone or enalapril. Although GFR is vulnerable to changes in both sodium and potassium balance, these results are consistent with the overall improvement in renal function and structure observed following co-treatment with either compound.

The reduction in renal toxicity observed following concomitant administration of enalapril and CsA contrasts, however, with the recent finding of Gerkens and Smith [23], who reported failure of the ACE inhibitor captopril (10 mg/kg/day) to influence CsA nephrotoxicity in Wistar rats on a low salt diet. In addition to differences in rat strain and salt diet between the two studies, however, Gerkens and Smith employed a higher dosage of CsA (100 mg/kg/48 hr) and did not measure PRA. Moreover, it has been reported that captopril is several fold less potent an ACE inhibitor than enalapril [24, 25].

This study is not the first to demonstrate that renin is released following the administration of CsA. Siegl *et al.* [13] and others [17–21, 26] have shown such an effect, which we have found to be partially dose-

Table 3. Effect of CsA alone or in combination with enalapril or spironolactone on plasma renin activity (ng angiotensin I/ml/hr)

| Day | Enalapril | Spironolactone | CsA | CsA + enalapril | CsA + spironolactone |
|---|---|---|---|---|---|
| 0 | 7.3 ± 2.9 | 6.0 ± 0.9 | 2.0 ± 1.4 | 7.6 ± 3.7 | 7.1 ± 2.3 |
| 4 | 15.7 ± 3.5† | ND | 11.7 ± 12.7† | 32.4 ± 6.0‡ | 25.5 ± 5.4* |
| 7 | 26.2 ± 6.1† | 10.3 ± 4.9† | 3.2 ± 1.8 | 26.0 ± 4.7‡ | 24.6 ± 5.9* |
| 14 | 24.7 ± 1.9‡ | 13.1 ± 5.4* | 4.3 ± 1.9 | 32.6 ± 6.5‡ | 23.2 ± 5.3* |

Results are expressed as the mean ± 1 SD and are compared to their corresponding pretreatment values by Student's *t*-test for paired samples: * $P < 0.01$; † $P < 0.05$; ‡ $P < 0.01$.
ND = not determined

Table 4. Effect of enalapril or spironolactone on CsA-induced proximal tubular damage

| Treatment | Proximal tubular damage score |
|---|---|
| CsA alone | 2.5 ± 0.5 |
| CsA + enalapril | 0.6 ± 0.6† |
| CsA + spironolactone | 1.5 ± 0.5 |

Results are expressed as the mean ± 1 SD and are compared to CsA alone using Student's *t*-test for unpaired samples: † $P < 0.05$. For scoring system see Materials and Methods.

dependent [21] and short-lasting. The current study is, however, the first to examine the potential role of the RAAS in the pathogenesis of the nephrotoxic effect of CsA by attempting to block both the effector arms of the system.

Renin release leads to the production of angiotensin II, both locally in the kidney and for release into the systemic circulation. Angiotensin II has two main effects—direct arterial vasoconstriction and modification of the renal distal tubular handling of water and electrolytes, the latter either via aldosterone released from the adrenal cortex or by direct local action in the kidney. Either or both of these effects could be involved in the nephrotoxicity of CsA. ACE-inhibition with enalapril will inhibit the conversion of angiotensin I to II, thus inhibiting both main effector arms of the RAAS, while spironolactone acts directly on distal tubular modification of urinary water and electrolytes, without affecting angiotensin II-mediated vasoconstriction. The sustained increases in PRA caused by treatment with enalapril or spironolactone indicate that blockade was being achieved, while the fact that animals treated only with enalapril or spironolactone showed no change in renal function despite high PRA, demonstrates that increased renin release and/or angiotensin I generation were not enough to cause the nephrotoxicity.

The protective effect of spironolactone cannot be attributed to its capacity to induce hepatic drug (CsA) metabolism and biliary flow [27], since no significant reduction in circulating CsA levels, as measured by radioimmunoassay were observed. Since enalapril and spironolactone reduced nephrotoxicity by more or less the same amount, it is likely that the aldosterone effector arm of the RAAS is associated with CsA nephrotoxicity. This is supported by the study of Siegl *et al.* [13], showing a doubling of plasma aldosterone levels in spontaneously hypertensive rats following CsA treatment for 4 weeks.

Most studies of CsA nephrotoxicity have shown that structural damage is confined to the proximal tubule [28], although Nemlander *et al.* [29] did claim distal tubular damage in normal rats given high doses of the drug. It is, however, possible to relate the proximal tubular damage to a distal tubular effect by way of the tubulo-glomerular feedback mechanism [30], which both we [12, 31] and others [10, 13] have postulated as being involved in CsA-induced nephrotoxicity. Early proximal tubular damage would reduce sodium reabsorption, thus increasing salt load in the distal tubule and thereby activating tubuloglomerular feedback with a consequent decrease in blood flow and GFR: our data suggest that it is the RAAS, specifically the aldosterone effector arm, which may mediate this feedback mechanism, but do not rule out a concomitant direct functional effect of CsA on the distal tubule.

Since the present experiment did not constitute

Table 5. Trough CsA levels

| Treatment | Day: μg/ml | | |
|---|---|---|---|
| | 4 | 7 | 14 |
| CsA alone | 4.98 ± 2.04 | 4.49 ± 3.39 | 6.45 ± 2.36 |
| CsA + enalapril | 4.08 ± 0.46 | 2.43 ± 1.07 | 4.20 ± 2.46 |
| CsA + spironolactone | 4.27 ± 0.48 | 5.65 ± 2.88 | 4.87 ± 1.48 |

Results are means ± 1 SD. There were 4 determinations per group on days 4 and 14 and 8 on day 7. There were no significant differences between the groups on any of the days studied.

a formal salt and water "balance" study, such an investigation must now be conducted to test conclusively our hypothesis regarding the role of the RAAS in the pathogenesis of CsA nephrotoxicity.

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