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EFFECT OF THROMBOXANE SYNTHETASE INHIBITION AND ANGIOTENSIN CONVERTING ENZYME INHIBITION ON ACUTE CYCLOSPORIN A NEPHROTOXICITY

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Abstract—One component of cyclosporin A (CsA) nephrotoxicity is thromboxane (Tx) A_2 induced renal vasoconstriction. This study was designed to investigate whether coadministration of angiotensin converting enzyme inhibitors (ACEI) and thromboxane synthetase inhibition (TSI) could act synergistically to improve the glomerular filtration rate in CsA treated animals. CsA administration (50 mg/kg/day p.o.) to Sprague–Dawley rats for 14 days caused a significant decline in creatinine clearance (CCR), an increase in *N*-acetyl-β-D-glucosaminidase (NAG) enzymuria and renal tubulointerstitial damage. These changes were associated with a ten-fold increase in urinary TxB_2 excretion (from pretreatment values of 17.2 ± 6.0 ng/day to 174.9 ± 65.4 ng/day on day 14). Treatment with TSI normalized TxB_2 excretion; this was associated with partial protection against CsA induced changes in CCR and NAG enzymuria and the complete prevention of acute proximal tubular vacuolation. However, the coadministration of both TSI and ACEI removed the protective effects exerted by TSI alone and resulted in elevated urinary TxB_2 levels similar to those observed in other CsA treated groups. Treatment with ACEI alone did not affect CsA nephrotoxicity. We suggest that elevated TxB_2 synthesis is in part responsible for some aspects of renal functional and morphological damage, but that CsA nephrotoxicity is multifactorial and may result from direct cellular toxicity in addition to vascular changes.

Acute cyclosporin A (CsA¶) nephrotoxicity is manifest functionally as a decrease in glomerular filtration rate (GFR). It is generally acknowledged that this effect is in part dependent on altered renal haemodynamics. Several studies in animals [1, 2] and in humans [3, 4] have revealed that CsA causes an increase in renal vascular resistance and a reduction in blood flow. Much of the interest in CsA nephrotoxicity has therefore focused on factors which normally control renal haemodynamics—eicosanoids, the renin–angiotensin system (RAS) and the renal sympathetic nervous system. The extent and nature of involvement of each of these factors in the development of toxicity is controversial. However, there is some evidence that CsA increases the levels of the vasoconstrictor eicosanoid, thromboxane (Tx) A_2, at least in rats [5, 6]; urinary TxB_2 levels increase after CsA treatment and the administration of thromboxane synthetase inhibitors has been shown to ameliorate toxicity [7, 8]. Such treatment, however, does not normalize the GFR, suggesting that additional factors are involved. Improved renal function after the administration of angiotensin converting enzyme inhibitors (ACEI), such as captopril [1] and enalapril [9], to CsA treated animals has also been observed although evidence for the involvement of angiotensin II (AII) in the pathogenesis of the toxicity and consequently the protective effects of ACEI are less well established. However, it has been shown (Ciba Geigy, personal communication) that an ACEI (CGS 16617) and a TSI (CGS 12970) when administered together to normotensive rats acted synergistically to reduce blood pressure.

Since functional CsA nephrotoxicity also has a haemodynamic basis, the aim of this study was to determine whether combining an ACEI and a TSI could produce a synergistic amelioration of the CsA induced reduction in GFR.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (250–350 g) obtained from Charles River U.K. Ltd (Margate, U.K.), were allowed free access to food and water throughout the experimental period.

Drugs. A stock solution of CsA (50 mg/mL) was prepared in ethanol:olive oil (Boots Company Ltd, Nottingham, U.K.; 1:9) and was administered to the conscious rat by gastric intubation using a No. 4 gauge cannula (Portex Ltd, Hythe, U.K.). Thromboxane synthetase inhibitor (CGS 12970; 8-[3-methyl-2-(3-pyridyl)-1-indolyl]-octanoic acid; Ciba Geigy, Horsham, U.K.) and ACEI (CGS 16617; 3-([5-amino-1-carboxy 1*S*-pentyl)]-amino)-2345 tetrahydro-2-oxo-3*S*-1*H*-1 benzazepine-1-acetic

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¶ Abbreviations: CsA, cyclosporin A; GFR, glomerular filtration rate; CGS 12970, 8-[3-methyl-2-(-3-pyridyl)-1-indolyl]-octanoic acid; CGS 16617, 3-([5-amino-1-carboxy 1*S*-pentyl]-amino)-2345 tetrahydro-2-oxo-3*S*-1*H*-1 benzazepine-1-acetic acid; PTV, proximal tubular vacuolation; CTD, chronic tubulointerstitial damage; Ca, calcification; CCR, creatinine clearance; NAG, *N*-acetyl-β-D-glucosaminidase; 4MU, methylumbelliferone; Tx, thromboxane; ACEI, angiotensin converting enzyme inhibitors; TSI, thromboxane synthetase inhibition; RAS, renin–angiotensin system.

Table 1. Treatment protocol

Group	CsA	TSI	ACEI	Saline	EtOH/Olive oil
CO	+	−	−	−	−
CT	+	+	−	−	−
CA	+	−	+	−	−
CAT	+	+	+	−	−
CS	+	−	−	+	−
U	−	−	−	−	−
VAT	−	+	+	−	+
VS	−	−	−	+	+

acid; Ciba Geigy) were suspended separately or together in NaCl (0.9%, pH > 9) to give a 2 mg/mL solution of each inhibitor which was administered as above.

Experimental protocol. There were eight groups of animals (Table 1). Five groups of six rats received CsA (50 mg/kg) either alone (CO) or in combination with TSI (CT) only, ACEI (CA) only, both inhibitors together (CAT) or with saline (CS). TSI and ACEI were administered daily at a dose of 10 mg/kg. A sixth group was left untreated (U) while the remaining two groups received ethanol:olive oil in combination with both inhibitors (VAT) or with saline (VS). Renal function and enzymuria were measured on days 0, 7 and 14.

Blood and urine sampling. Animals were placed in individual metabolic cages overnight (16–24 hr) and urine free from faecal contamination was collected at ambient temperature. Blood samples (2 mL) were obtained by tail clipping under light anaesthesia and were placed in tubes containing lithium heparin as an anticoagulant.

Biochemical investigations. Heparinized blood samples were spun at 4000 rpm for 10 min and the plasma removed for measurement of creatinine using a SMAC Analyser (Technicon Instruments Ltd, Tarrytown, U.S.A.). Estimations of creatinine and glucose in urine were performed using a Technicon RA-1000 Analyser and a Beckman Glucose Analyser (Beckman RICC Ltd., Glenrothes, U.K.), respectively. Urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity was measured as previously described [10] and was expressed as nmol 4MU released per hour per mmol of urinary creatinine, a measure independent of urine flow rate (UFR).

Measurement of CsA. Trough CsA concentrations were measured in whole blood on day 14 by radioimmunoassay using kits supplied by Sandoz Ltd (Basle, Switzerland). The antibody does not distinguish between the parent CsA molecule and certain metabolites.

Measurement of urinary thromboxane B_2. Thromboxane B_2 levels were measured in urine diluted 10-fold using a specific monoclonal radioimmunoassay kit based on magnetic separation (Amersham International, Amersham, U.K.).

Histology. At the end of the experimental period, rats were killed by cervical dislocation under ether anaesthesia. The kidneys were removed, sectioned coronally and fixed in formalin. Sections (5 μm thick) stained with haematoxylin and eosin were randomized and examined blind under light microscopy for the presence of drug induced changes, particularly proximal tubular vacuolation (PTV), chronic tubulointerstitial damage (CTD) and calcification (Ca). Each feature was assessed on a scale from 0 to 4, where 0 was no abnormality and 1, 2, 3, and 4 represented mild, moderate, moderately-severe and severe abnormalities, respectively.

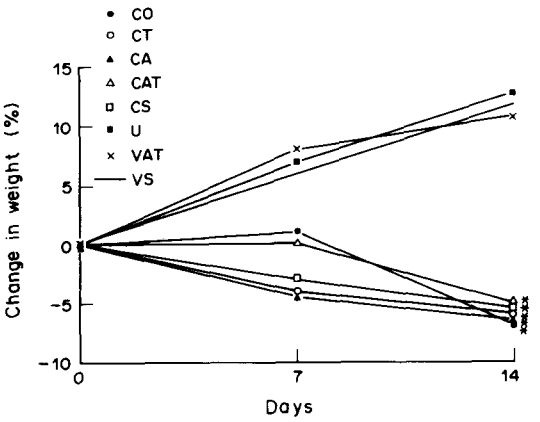

Fig. 1. Change in weight (%) in control and CsA treated animals. Results are expressed as means. Significant differences ($P < 0.01$) at day 14 assigned using Dunnett's test are denoted as * for untreated (U) vs all other groups.

Statistics. For multiple comparisons, results were compared by one way or two way analysis of variance where appropriate with significances assigned using Neuman–Keul's test. For single comparisons, results were compared using an unpaired *t*-test.

RESULTS

Administration of CsA (50 mg/kg) for 14 days to Sprague–Dawley rats resulted in weight loss (1.2 g day) which was similar in all treatment groups; normal weight gain (8 g/day) was observed in animals receiving drug vehicles or inhibitors (Fig. 1).

CsA treatment alone (CO) resulted in a progressive decrease in CCR (Fig. 2) which was evident at day 7 and significantly different ($P < 0.05$) from control values (VS, VAT, U) at day 14. A similar change in renal function was observed after the first week in animals additionally receiving TSI (CT). Thereafter, there was no further reduction in renal function and on day 14, CCR was significantly higher ($P < 0.05$) in this group compared to two other CsA treated groups (CO, CS). Furthermore, although CCR was lower in group CT, it was not significantly different from that of control groups U and VAT. In contrast, the coadministration of ACEI with TSI (CAT) appeared to remove the protective effect exerted by TSI alone. No improvement in CCR was observed due to ACE inhibition (CA) or saline (CS).

A similar profile was observed for the change in NAG enzymuria (Fig. 3) in the CsA treated groups over the 14 day period: there was a progressive rise in NAG activity in animals receiving CsA only but levels stabilized after day 7 in those additionally receiving TSI. At the end of the study, NAG enzymuria was significantly lower in group CT than in other

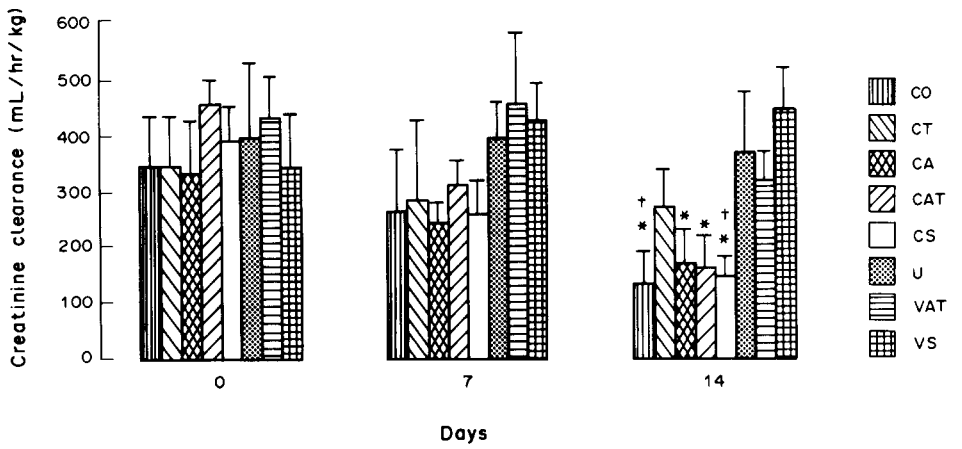

Fig. 2. The effect on CCR of administering CGS 12970 and CGS 16617 to CsA treated animals. Results are expressed as means ± SD. Significant differences ($P < 0.05$) at day 14 assigned using Neuman–Keul's test are denoted as * for control vs CsA treated groups and as † for CT vs other CsA treated groups.

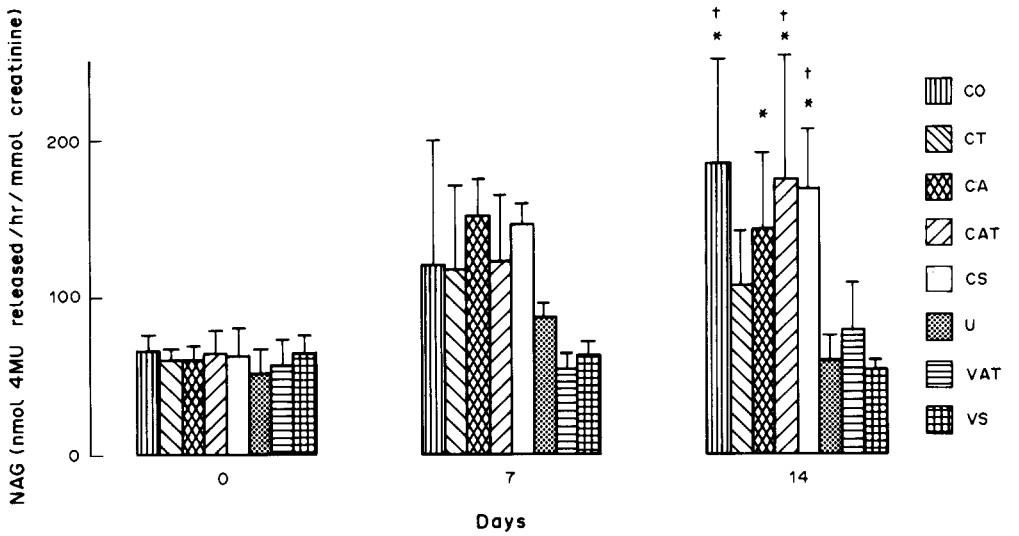

Fig. 3. The effect on NAG enzymuria on days 0, 7 and 14 of administering CGS 12970 and CGS 16617 to CsA treated animals. Results are expressed as means ± SD. Significant differences ($P < 0.05$) at day 14 assigned using Neuman–Keul's test are denoted as * for control vs CsA treated groups and as † for CT vs other CsA treated groups.

CsA treated groups (CO, CAT, CS). Neither treatment with drug vehicles (VS) nor the administration of inhibitors to vehicle treated animals (VAT) had any effect on CCR or NAG enzymuria.

Glycosuria (Fig. 4) and UFR (Table 2) were elevated in all CsA treated groups on days 7 and 14. No protective effect was observed in response to the administration of either inhibitor given alone or in combination. The change in both parameters was greatest on day 7 with mean group values of 15.58 ± 7.04 mmol/mmol urinary creatinine (Ucr) and 3.17 ± 0.33 mL/hr/kg compared with pretreatment values of 0.47 ± 0.07 mmol/mmol Ucr and 1.61 ± 0.15 mL/hr/kg for glycosuria and UFR, respectively. On day 14 there was a moderation in the magnitude of change in both indices and the level of glycosuria but not of UFR was significantly higher in all CsA treated groups compared with control animals. Levels of glucose in the blood of CsA treated rats (10.3 ± 1.3 mmol/L) and control rats (9.3 ± 1.5) were not significantly different suggesting that glycosuria was not due to an increased filtered load.

Coadministration of ACEI and TSI alone or in combination and of saline did not alter the circulating levels of CsA measured in whole blood after 14 days of treatment (Table 3). The differences in renal function observed between CsA treatment groups were therefore not due to differences in drug concentration.

CsA treatment also caused three distinct types of morphological lesion: proximal tubular vacuolation

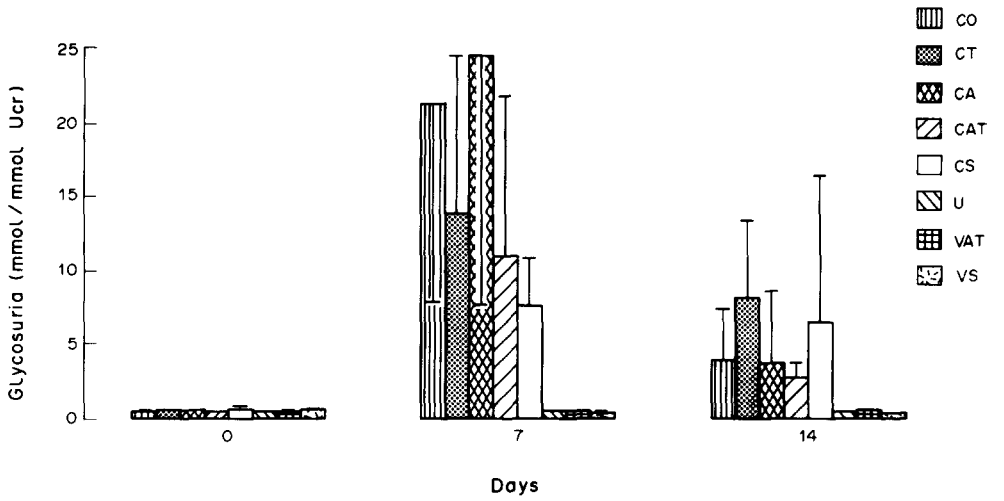

Fig. 4. The effect of CsA treatment on glycosuria on days 0, 7 and 14. Results are expressed as means ± SD.

Table 2. Effect of CSA on urine flow rate

Group	Urine flow rate (mL/hr/kg) 0	7	14
CO	1.71 ± 0.51	3.07 ± 0.89	2.35 ± 2.16
CT	1.77 ± 0.50	3.70 ± 1.58	2.83 ± 1.13
CA	1.46 ± 0.38	2.91 ± 0.88	2.58 ± 0.49
CAT	1.54 ± 0.38	2.92 ± 0.83	2.10 ± 1.25
CS	1.72 ± 0.32	3.24 ± 1.02	3.07 ± 1.37
U	1.77 ± 0.64	1.39 ± 0.43	1.47 ± 0.45
VAT	1.48 ± 0.60	2.14 ± 0.62	2.04 ± 0.71
VS	1.43 ± 0.25	1.62 ± 0.45	1.62 ± 0.66

Results are expressed as mean ± SD.

Table 3. Trough CSA levels in whole blood on day 14

Group	CSA levels (μg/mL)
CO	5.0 ± 1.4
CT	4.2 ± 1.2
CA	5.0 ± 0.5
CAT	5.0 ± 0.7
CS	3.9 ± 0.4

Results are expressed as means ± SD.

(PTV), chronic tubulointerstitial damage (CTD) and calcification (Ca). PTV was confined to the cells of the straight proximal tubule. This abnormality was recorded in some animals of all CsA treated groups (Table 4) except in those additionally receiving TSI where its absence suggested a protective role for the inhibitor. However, the degree of morphological damage in the other groups was highly variable; some rats were unaffected whilst in others large numbers of tubule cells contained cytoplasmic vacuoles. Varying degrees of CTD and Ca, affecting the cortex and the corticomedullary junction, respectively, were evident in all CsA treated groups. Whereas virtually all animals showed some chronic damage, calcification was a more variable feature of CsA treatment. No structural abnormalities were present in the control groups.

Table 4. Morphological indices of CSA nephrotoxicity

Group	PTV	CTD	Ca
CO	2.5 ± 1.8	2.0 ± 0.9	1.3 ± 1.5
CT	0 ± 0	2.0 ± 1.1	0.7 ± 0.8
CA	1.2 ± 1.8	1.7 ± 1.4	0.5 ± 0.8
CAT	2.8 ± 1.8	1.8 ± 1.0	0.3 ± 0.8
CS	1.2 ± 1.8	2.0 ± 0.6	1.2 ± 1.2
U	0 ± 0	0 ± 0	0 ± 0
VAT	0 ± 0	0 ± 0	0 ± 0
VS	0 ± 0	0 ± 0	0 ± 0

Results are expressed as means ± SD.

CsA (CO) treatment for 14 days caused a 10-fold increase in the excretion of immunoreactive thromboxane B_2 whereas coadministration of TSI alone completely prevented this rise (Fig. 5). When both inhibitors were given together to CsA treated animals (CAT), the inhibitory effect of TSI was lost. Interestingly, the coadministration of both inhibitors to vehicle treated rats (VAT) resulted in a reduction ($P < 0.01$) in Tx excretion on day 14 compared with values observed in untreated (U) and vehicle treated (VS) animals.

Neither the inhibition of ACE nor saline administration had any effect on the CsA induced increase in urinary Tx levels.

DISCUSSION

One major pathogenic effect of CsA is TxA_2 mediated renal vasoconstriction which results in a reduction in GFR. Evidence derived mainly from

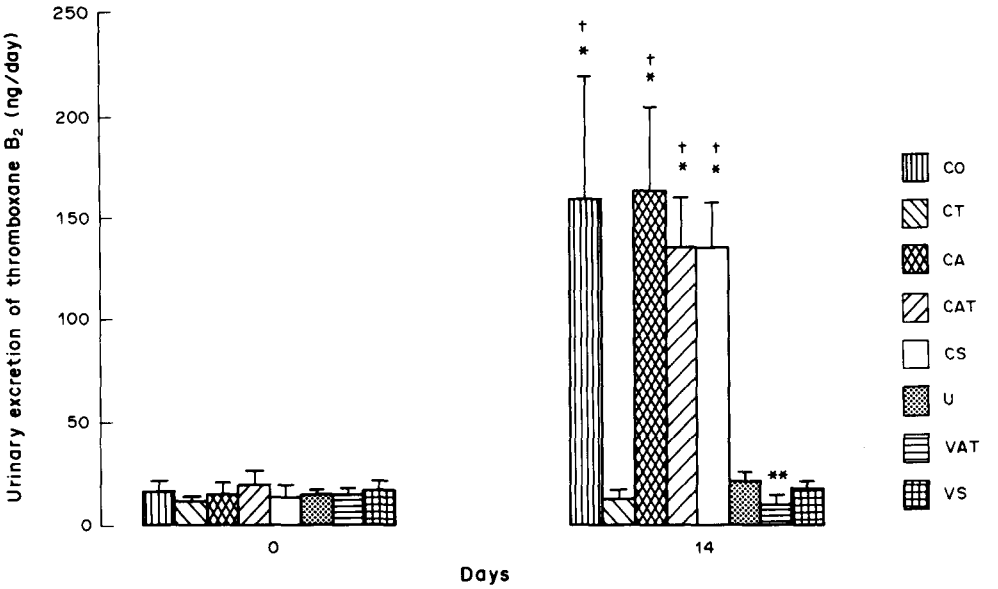

Fig. 5. The effect of CGS 12970 and CGS 16617 on the excretion of urinary TxB_2 in CsA treated animals on days 0 and 14. Results are expressed as means ± SD. Significant differences ($P < 0.05$) at day 14 assigned using Neuman–Keuls test are denoted as * for controls (VS, VAT, U) vs CsA treated groups and as † for CT vs other CsA treated groups. ** Denotes a significant difference ($P < 0.05$, Student's *t*-test) between VAT vs U and VS.

animal models suggests that CsA treatment is accompanied by a gradual rise in urinary TxB_2 excretion which precedes the deterioration in renal function [5, 6]. A close correlation has also been observed between TxB_2 excretion and plasma CsA [6] levels whilst Perico *et al.* [8] showed an inverse correlation between TxB_2 and GFR.

In this study the significant reduction in CCR on day 14 in CsA treated rats was accompanied by a 10-fold increase in urinary TxB_2 excretion. Administration of CGS 12970 (10 mg/kg) successfully prevented elevated excretion and the normalized levels of TxB_2 on day 14 were associated with a marked improvement in CCR. TxA_2 may compromise glomerular haemodynamics in several ways. The eicosanoid has been shown to have a direct contractile effect on renal mesangial cells [11, 12] in culture, which would thus reduce the glomerular ultrafiltration rate [13, 14] *in vivo*. In addition, English *et al.* [15] using CsA at a dose of 50 mg/kg demonstrated that the drug caused a dramatic reduction in the diameter of the afferent arterioles. It is possible therefore, that TxA_2 predominantly affects the afferent arterioles and that TSI improves GFR in CsA treated animals by preventing TxA_2 constriction at this site. However, it has also been demonstrated that TSI can result in an accumulation of prostaglandin endoperoxides capable of being metabolized to vasodilatory products [16, 17]. Thus, it cannot be excluded that the beneficial effect of TSI may, in part, relate to increased synthesis of vasodilatory prostanoids rather than to selective inhibition of Tx formation.

The inability of TSI to restore CCR to levels comparable with control animals despite the normalization of TxB_2 implies the involvement of other factors. These may include CsA induced stimulation of the renal sympathetic nervous system [2, 18], or alterations in the synthesis of vasodilatory prostaglandins [19]. However, the possibility that the renin–angiotensin system is a contributory factor in the development of nephrotoxicity is unlikely. Administration of CGS 16617 at a dose of 10 mg/kg did not improve CCR despite previous observations [20] that the dicarboxylic acid is orally bioavailable and has an IC_{50} for ACEI of 1.7 mg/kg in rats. Furthermore, AII has been shown to cause contraction of the efferent post-glomerular arterioles [21, 22] and therefore could not be responsible for the afferent arteriolar vasoconstriction associated with CsA [15].

When CGS 12970 was given in combination with ACEI, the urinary excretion of TxB_2 was greatly enhanced on day 14 and correspondingly, the protective capability of TSI was lost. This result was unexpected and contradicts the suggestion that dual administration of the inhibitors would bring about synergistic amelioration of CsA induced nephrotoxicity. One possible explanation for this is that TSI and ACEI effectively vasodilate the afferent and efferent renal arterioles, respectively. This may reduce the glomerular filtration pressure to a critical level causing severe depression of GFR. To re-establish vascular resistance and filtration pressure, the kidney may respond by greatly enhancing TxA_2 synthesis thereby overriding the inhibitory effect of TSI. This theory necessitates that the kidney has a large reserve capacity to synthesize TxA_2. The administration of both inhibitors to vehicle treated rats had no effect on TxB_2 levels or on CCR suggesting that dual treatment exerts a pathogenic effect only when intrarenal control of GFR is already lost by CsA treatment [23].

There is little disagreement that CsA nephrotoxicity compromises renal function and that renal vasoconstriction is a major causal factor. However, the possibility that tubulotoxicity contributes to the functional lesion remains controversial. In this study, and in others [24], CsA stimulated an increase in UFR and glycosuria which suggests impairment in the ability of the proximal tubule to reabsorb filtered water and glucose.

CsA also caused a time-dependent increase in the urinary excretion of the predominantly proximal tubular enzyme *N*-acetyl-β-D-glucosaminidase. McAuley *et al.* [25] have found very early changes in the level of urinary NAG in CsA treated animals which preceded both renal functional and structural damage; NAG enzymuria may therefore be a sensitive index of proximal tubular integrity and could serve as a useful adjunct to the diagnosis of CsA nephrotoxicity [25].

In addition to the degeneration in tubular integrity, CsA caused histological abnormalities. Proximal tubular vacuolation is regarded as the classic experimental feature of acute CsA toxicity. As was observed in this study and has been reported elsewhere [26], vacuolation may be prevented by pharmacological intervention; the administration of TSI alone to CsA treated animals completely protected against the development of PTV on day 14. However, PTV is cyclical rather than progressive in nature and may also reverse spontaneously [27]. This may account for the observed variability in the severity and incidence of the lesion in CsA treated animals. These characteristics also bring into question the significance of vacuolation as a specific indicator of proximal tubular integrity. CTD and corticomedullary calcification were similar to the histological changes previously reported [27, 28]. In contrast to PTV, the development of these abnormalities is progressive [27] and at least in this study neither was affected by TSI administration. There was no relationship between the degree of CTD and of calcification with PTV which suggests that the two types of lesion have a separate causal mechanism.

Whilst CsA undoubtedly causes degenerative changes in the renal tubules it is unclear whether these result from direct drug toxicity or are secondary to vascular changes. Numerous reports based on *in vitro* experimentation suggest that CsA is directly toxic to some types of kidney cell. With particular relevance to this study, Scoble *et al.* [29], showed that CsA prevented the uptake of glucose by cultured LLC-PK cells, a cell line which possesses proximal tubule characteristics; this suggests that glycosuria observed *in vivo* in this study may be due to direct CsA toxicity. Moreover, TSI did not ameliorate the magnitude of glycosuria despite an improvement in CCR. In contrast, however, the administration of TSI alone but not in combination with ACEI resulted in a moderation in the degree of NAG enzymuria and the complete prevention of PTV. These tubular changes were related to corresponding changes in CCR in both groups of animals which suggests that the protective effects were secondary to an improvement in renal blood flow.

In this study acute CsA nephrotoxicity was assessed in terms of alterations in renal function and morphology. TSI administration ameliorated some aspects of CsA nephrotoxicity but these protective effects were lost by cotreatment with TSI and ACEI. The manifestation of protection and its loss were not due to differences in the circulating levels of CsA since inhibitor treatment did not influence the trough drug concentration. Although it seems reasonable to conclude that an increase in TxA_2 is partly responsible for the decrease in GFR, the lesion in general, is poorly understood. Experimental studies which have attempted to prevent CsA induced damage by improving renal haemodynamics have at most modified the attendant nephrotoxicity. Further work is therefore required to elucidate the relevance of tubular effects to compromised GFR and to define the inter-relationship between functional and structural toxicity.

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