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THE ROLE OF EICOSANOIDS IN CYCLOSPORINE NEPHROTOXICITY IN THE RAT

A. ERMAN,* B. CHEN-GAL and J. ROSENFELD

Institute of Nephrology and Hypertension, Beilinson Medical Center, Petah Tikva, 49100, Israel, and Sackler School of Medicine, Tel Aviv University, Israel

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Abstract—Nephrotoxicity is the most troublesome complication of cyclosporine (CSA) therapy. The present study was designed to investigate the effects of chronic treatment with CSA on the 24-hr urinary excretion of prostanoids (PGs) and thromboxane (Tx) and on the renal function in the absence or presence of indomethacin. CSA administration to Wistar rats (20 mg/kg/day, i.p.) for 14 days caused a significant increase in plasma creatinine, blood urea nitrogen (BUN), urine osmolality, fractional excretion of sodium and potassium and a reduction in creatinine clearance (CCr) and urine volume. These changes were associated with a significant reduction in urinary excretion of PGE2 (21.1 ± 3.3 vs 33.0 ± 2.5 ng/24 hr) and PGF2α (13.4 ± 1.4 vs 27.9 ± 3.8 ng/24 hr) and an increase in TxB2 (12.1 ± 3.0 vs 4.6 ± 0.5 ng/24 hr), and 6-keto PGF1α (56.2 ± 7.7 vs 27.7 ± 1.9 ng/24 hr). However, the synthesis of TxB2 and 6-keto PGF1α by renal medullary and cortical slices prepared from CSA treated rats was not different from values obtained for vehicle treatment. In contrast, PGE2 synthesis by cortical slices prepared from the CSA group was increased. A single injection of indomethacin (10 mg/kg) to vehicle and CSA treated rats resulted in a significant reduction in PGs and TxB2 excretion. This, was associated with a further reduction in CCr (0.81 ± 0.06 vs 1.03 ± 0.04 ml/min) and an increase in BUN (38.5 ± 5.2 vs 28.2 ± 1.4 mg%) only in the CSA group. We suggest that the vasodilating PGs attenuate the renal toxic effects induced by CSA.

Nephrotoxicity is the most significant complication of cyclosporine (CSA) therapy. Treatment with CSA may result in a decrease in glomerular filtration rate, renal blood flow, urine flow and body weight in laboratory animals [1]. Although CSA treatment causes activation of the renin-angiotensin system [2] and suppression of the prostaglandin system [3, 4] in the rat, the biochemical mechanisms underlying these nephrotoxic effects are still unclear.

The hypothesis that CSA-induced nephrotoxicity is mediated by prostanoids was suggested [5]. Indeed, in the rat the administration of the stable analog of PGE2,16,16-dimethyl PGE2 have been demonstrated to prevent the CSA-induced nephrotoxicity [6, 7]. Several studies have reported that renal PGs are depressed by CSA [8, 9]. In contrast Murray *et al.* observed that CSA treatment is associated with an increase in the excretion of 6-keto prostaglandin F1α [10] and an increase in the excretion of thromboxane B2 (TxB2) a stable metabolite of thromboxane A2 [11]. Inhibition of PGs synthesis by meclofenamate [10] or indomethacin [12] led to an increase in CSA nephrotoxicity, but recently Barros *et al.* reported that indomethacin did not alter the effect induced by CSA treatment [13].

The conflicting and inconclusive studies do not permit conclusions to be drawn concerning the role of PGs in CSA nephrotoxicity. Therefore, the present study was designed to investigate the effect of chronic treatment with CSA on the 24 hr urinary excretion of PGs and TxB2 and on renal function with and without indomethacin.

* Correspondence should be addressed to Dr A. Erman, Renal and Hypertension Unit, Beilinson Medical Center, Petah Tikva, 49100, Israel.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats (bred at the Beilinson Medical Center, Israel) weighing 250–270 g. The rats were kept in group cages and had free access to tap water and were fed *ad lib.* a standard chow (Purina). The studies were performed after 4 days of acclimatization of the rats. The rats were injected with CSA 20 mg/kg/day (i.p.) or its vehicle (10% ethanol in olive oil), for 14 days. At day 14 the rats were housed in individual metabolic cages and 24-hr urine collections were obtained and kept frozen in the presence of indomethacin (10 μg/ml) until analysed for PGs. On day 15, rats were anaesthetized with ether, the abdominal aorta was punctured and 4–6 ml blood was collected for the determination of creatinine, blood urea nitrogen (BUN) and potassium. The kidneys were removed immediately for the preparation of renal medullary and cortical slices.

Slices of renal inner medulla and cortex from vehicle and CSA treated rats were incubated in a final volume of 1.5 ml for 30 min at 37° in Krebs buffer containing 10 mM Tris–HCl (pH 7.4). At completion of the incubation the medium was removed and kept frozen until PGs were determined in the unextracted samples and the slices were dried to constant weight. Results are expressed as nanograms of immunoreactive eicosanoids released during 30 min incubation per milligram dry weight.

The excretion of PGs (PGE2, PGF2α, 6-K-PGF1α) and TxB2 was determined in the 24-hr urine specimens by RIA method after lipid extraction and

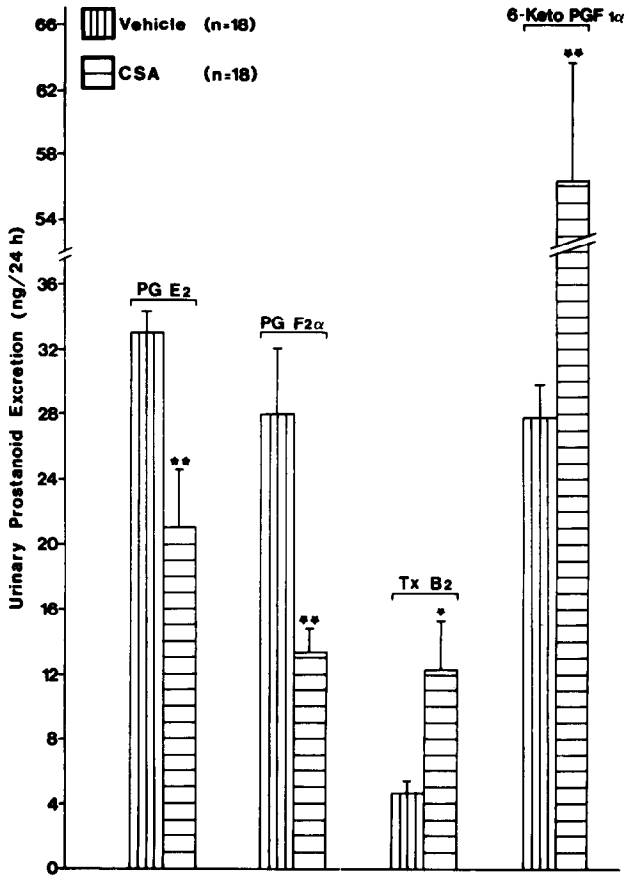

Fig. 1. Effect of CSA treatment for 14 days on urinary excretion of immunoreactive prostanoids and thromboxane. Rats received injections of CSA (N = 18) or its vehicle, olive oil with 10% ethanol (N = 18) for 14 days. PGs and TxB2 were determined in 24-hr urine collections by RIA as described in Materials and Methods. Values are mean ± SE. The data was analysed by Student's *t*-test. * $P < 0.02$, ** $P < 0.01$.

purification as described previously [14]. Briefly, two milliliters of urine were acidified to pH 3.0–3.5 with 2 M citric acid and extracted twice with two volumes of ethyl acetate. The organic phase was evaporated and the lipid extract was applied to silica-gel G plates (Merck, Darmstadt, F.R.G.) and chromatographed concurrently with authentic PGs and TxB2 standards using as a solvent system the organic phase of ethyl acetate–isooctane–acetic acid–water (11:5:2:10). The zones on the plate corresponding to the position of 6-keto PGF1α, PGF2α, PGE2 and TxB2 were scraped and the eicosanoids were eluted with methanol. The methanol was evaporated to dryness under a nitrogen stream and the eicosanoids were resuspended in 1 ml phosphate buffer (0.01 M, pH 7.4) containing 0.15 M NaCl, 0.1% NaN_3 and 0.1% BSA and measured by RIA. The overall recovery of the various PGs and TxB2 in the course of extraction and separation was 45–60% as determined by the addition of radioactive tritiated PGs and TxB2 to the urine before extraction. These recovery values served to correct the urine eicosanoid measurements. The urinary excretion of the different eicosanoids was expressed as nanograms of immunoreactive eicosanoid excreted per 24 hr.

Plasma and urine creatinine were measured using a Beckman creatinine analyser and BUN was measured using SMA analyser (Technicon, Tarrytown, U.S.A.). Serum and urine sodium and potassium concentrations were measured by flame photometry (Instrumentation Laboratory, 943, Milan, Italy). Urine osmolality was determined by the freezing point method (Osmotte A). Creatinine clearance (CCr) was calculated as the amount of creatinine excreted in the urine per min divided by its concentration in the plasma, and fractional excretion (FE) of sodium and potassium were calculated as the percentage of the amount of cation excreted per min divided by its concentration in the plasma and the glomerular filtration rate, as reflected by CCr.

CSA as a pure powder was a generous gift of The Sandoz Company (Basel, Switzerland). PGs were purchased from Sigma (Petah Tikva, Israel). Rabbit anti PGE2, anti PGF2α, anti 6-K-PGF1α and rabbit anti TxB2 were obtained from Bioyeda (Rehovot, Israel). Tritiated radioactive PGs: PGE2 (160 Ci/mmol), PGF2α (177 Ci/mmol), 6-K-PGF1α (150 Ci/mmol) and TxB2 (210 Ci/mmol) were purchased from Amersham (Buckinghamshire, England).

Table 1. Effect of CSA administration on plasma creatinine, creatinine clearance, BUN, plasma potassium, urine volume and osmolality, sodium and potassium excretion

	Vehicle (N = 18)	CSA (N = 18)	P value
Plasma creatinine (mg/dl)	0.48 ± 0.02	0.65 ± 0.03	<0.001
CCr (ml/min)	1.34 ± 0.06	0.73 ± 0.07	<0.001
BUN (mg/dl)	21.9 ± 0.77	31.7 ± 3.1	<0.001
Plasma K+ (mEq/l)	4.54 ± 0.12	4.93 ± 0.08	<0.01
UV (ml)	32.3 ± 2.1	17.1 ± 2.9	<0.001
UNaV (mEq/day)	0.68 ± 0.006	0.66 ± 0.06	N.S.
FENa (%)	0.24 ± 0.03	0.45 ± 0.04	<0.01
UKV (mEq/day)	1.47 ± 0.06	1.39 ± 0.10	N.S.
FEK (%)	16.8 ± 1.5	26.8 ± 2.0	<0.01
Osmolality (mOsm/kg H_2O)	496 ± 29	970 ± 72	<0.001

Rats received injections of CSA (20 mg/kg/day) or vehicle (10% ethanol in olive oil) for 14 days. On day 14 the rats were housed in metabolic cages for 24-hr urine collection. On day 15, blood was withdrawn. Values are mean ± SE. Statistical significance was tested using unpaired Student's *t*-test.

Statistical analysis was performed using the Statistical Package for Social Sciences. Comparisons of difference between two experimental groups were tested using the Student's *t*-test and the least significant difference (LSD) method was used for multiple group comparisons. A P value less than 0.05 was considered significant.

RESULTS

CSA administration to Wistar rats (20 mg/kg/day i.p.) for 14 days caused a significant increase in plasma creatinine, blood urea nitrogen, hyperkalemia and a reduction in creatinine clearance (Table 1). These parameters were not different in the vehicle-treated rats compared to untreated rats. In addition, CSA treatment resulted in a 50% decrease in urine volume ($P < 0.001$) and about two-fold increase in urine osmolality ($P < 0.001$). The total excretion of sodium and potassium was not changed, however, the fractional excretion of these electrolytes was significantly increased (87.5, 59.5% accordingly, Table 1). Rats that were injected with CSA weighed on day 14 significantly less ($P < 0.05$) than the vehicle treated group (259.8 ± 5.8 vs 280.6 ± 5.8 g).

CSA treatment resulted in alterations in the PGs and TxB2 excretion. The urinary excretion of PGE2 and PGF2α was significantly reduced by 36 and 52% accordingly whereas the excretion of TxB2 and of 6-K-PGF1α was increased by 163 and 103%, accordingly (Fig. 1). The basal synthesis and release of thromboxane and prostacyclin by renal medullary and cortical slices prepared from CSA-treated rats was not different from values obtained for the vehicle treated group. In contrast, PGE2 synthesis by cortical slices prepared from CSA group was significantly increased ($P < 0.05$, Table 2).

Single injection of indomethacin to vehicle or CSA-treated rats (10 mg/kg) resulted in a significant reduction of urinary PGs and TxB2 excretion. In the CSA group, indomethacin treatment caused a further significant reduction in PGE2 and PGF2α excretion (44, 92% accordingly) and abolished the increase in thromboxane and prostacyclin synthesis and excretion (Fig. 2). These changes were associated with a further deterioration in kidney function. Indomethacin treatment caused a small, but insignificant, increase in plasma creatinine and BUN in the vehicle group, whereas in the CSA-treated rats indomethacin treatment resulted in a further increase in plasma creatinine and BUN and a decrease in creatinine clearance (Table 3). Indomethacin did not affect significantly urine volume or electrolytes excretion although in the CSA group there was a tendency of decrease in sodium (0.48 ± 0.09 vs 0.67 ± 0.12 mEq/day) and potassium (1.19 ± 0.14 vs 1.55 ± 0.15 mEq/day) excretion.

DISCUSSION

Nephrotoxicity is the most significant clinical side effect of CSA and was reported following kidney, liver and heart transplants [1, 15]. CSA administration to rats in a dose of 20 mg/kg/day (i.p.) for 14 days induced renal toxicity which was associated with alterations in eicosanoids excretion. The excretion of PGE2 and PGF2α was reduced, while that of TxB2 and 6-keto PGF1α was increased. In agreement with these findings other investigators have reported that CSA administration to conscious rats was associated with a significant increase in 6-keto PGF1α excretion [10] and an increase in TxB2 excretion [10, 16]. However, these effects on eicosanoids excretion are different from its effects on renal PGs biosynthesis in few different *in vitro* settings. For example, CSA treatment did not affect the synthesis of PGI2 and PGE2 by isolated rat glomeruli [17] or the formation of PGs by rat renal cortical slices [18]. In contrast, Stahl and Kudelka have demonstrated that chronic CSA treatment resulted in a reduction in PGE2 formation in isolated glomeruli and papilla of rat kidneys [19]. We observed that the synthesis and release of 6-keto PGF1α and TxB2 by medullary and cortical slices prepared from CSA-treated rats, were not changed. However, the synthesis of PGE2 by cortical slices was significantly increased. Therefore, eicosanoids

Table 2. Effect of CSA treatment on prostanoid production by medulla and cortical slices

Tissue	Treatment		Prostanoid production (ng/mg dry weight/30 min) Tx B_2	6-Keto $PGF_{1\alpha}$	PGE_2
Medulla	Vehicle	(N = 10)	2.4 ± 0.2	22.8 ± 3.3	59.1 ± 11.0
	CSA	(N = 10)	2.0 ± 0.2	21.3 ± 3.2	61.0 ± 11.7
Cortex	Vehicle	(N = 10)	0.027 ± 0.003	0.027 ± 0.003	0.032 ± 0.003
	CSA	(N = 10)	0.028 ± 0.004	0.032 ± 0.005	0.079 ± 0.018*

Slices of renal inner medulla and cortex from vehicle and CSA-treated rats were incubated for 30 min at 37° in Krebs–Tris buffer (pH 7.4), and PGs were determined by RIA on the unextracted media. Values are mean ± SE. * $P < 0.05$.

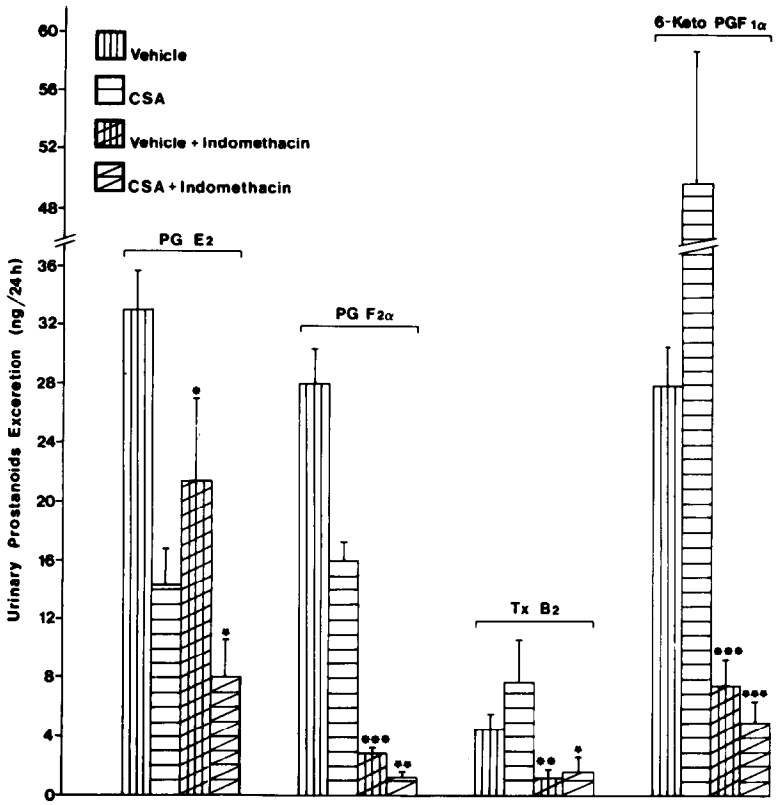

Fig. 2. Effect of indomethacin administration to CSA-treated rats on urinary excretion of prostanoids and thromboxane. Rats treated with CSA or vehicle received on day 14 a single injection of indomethacin (10 mg/kg) followed by 24-hr urine collection. Each group consists of 10 rats. PGs and TxB2 were determined by RIA as described in Materials and Methods. Values are mean ± SE. For statistical analysis the CSA + indomethacin group was compared to the CSA group and the vehicle group was compared to the vehicle + indomethacin group using Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

metabolism *in vitro* may not entirely reflect the synthetic and catabolic activity in the *in vivo* setting.

The origin of 6-keto PGF1α appearing in the urine might originate from intrarenal [20] and extrarenal sources [21]. It was observed that CSA impairs vascular PGI2 formation *in vivo* [4], inhibits PGI2 synthesis by cultured endothelial cells [3] and by macrophages [22]. Therefore, it could be speculated that the increase in 6-keto PGF1α excretion due to CSA treatment reflects an enhanced intrarenal synthesis of PGI2. The acute and chronic administration of CSA to rats stimulates intrarenal release and activity of renin [2, 10]. Since angiotensin II stimulates the synthesis of PGI2 [23] it is conceivable

Table 3. Effect of indomethacin administration to CSA-treated rats on plasma creatinine, creatinine clearance, BUN and urinary volume

Group	Plasma creatinine (mg/dl)	CCr (ml/min)	BUN (mg/dl)	UV (ml)
Vehicle (N = 14)	0.45 ± 0.02	1.36 ± 0.09	16.6 ± 1.3	33.9 ± 4.5
CSA (N = 14)	0.52 ± 0.02	1.03 ± 0.04	28.2 ± 1.4	21.2 ± 4.5
Vehicle + I (N = 14)	0.49 ± 0.02	1.26 ± 0.07	19.7 ± 1.0	34.0 ± 3.6
CSA + I (N = 14)	0.66 ± 0.04	0.81 ± 0.06	38.5 ± 5.2	18.9 ± 4.0
P value V-I vs V	N.S.	N.S.	N.S.	N.S.
P value CSA-I vs CSA	<0.01	<0.05	<0.05	N.S.
P value CSA-I vs V-I	<0.01	<0.01	<0.01	<0.05

Vehicle (V) and CSA-treated rats received, on day 14, a single injection of indomethacin (I, 10 mg/kg, S.C.) and were housed in metabolic cages for 24-hr urine collection. Values are mean ± SE. Statistical significance was tested using the least significant difference method for multiple group comparisons.

that this activation of the renin-angiotensin system may lead to enhanced intrarenal synthesis of PGI2 and an enhanced excretion of its metabolites. This enhanced production of the vasodilatory PGI2 may counteract the vasoconstrictory effects induced by angiotensin II and the increased levels of TxB2.

Inhibition of PGs and TxB2 synthesis by indomethacin as reflected by a decreased excretion, was associated with a deterioration in kidney function only in the CSA-treated rats, but not in the vehicle group. This observation is in agreement with the findings that following meclofenamate or indomethacin, rats treated with CSA demonstrated a further reduction in renal blood flow and an increase in renal vascular resistance [10] and an increase in BUN and serum creatinine [12]. However, it contradicts the observation of Barros *et al.* demonstrating that indomethacin failed to modify the renal alterations induced by CSA in the rat [13]. The beneficial effect and the protective properties of PGE2 and PGI2 in various tissues [24–26] including the kidney [27] during hypoxia, ischemia and mucosal damage have been demonstrated. This study demonstrates that CSA treatment resulted in an increase in 6-keto PGF1α and TxB2 excretion and a reduction in that of PGE2. In addition, the inhibition of PGs and TxB2 synthesis by indomethacin was associated with further deterioration in kidney function only in CSA-treated rats. Therefore, we suggest that the vasodilatory PGs, prostacyclin and PGE2, play a significant role in attenuating the renal toxic effects induced by CSA.

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