

## From Bedside to Bench Drug-induced Tubulointerstitial Disease Cyclosporine Nephropathy Study from Models of Cultured Renal Epithelial Cells

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Cyclosporine (CsA) is a potent immunosuppressant used in the prevention of transplanted organ rejection. CsA is associated with sodium retention, hypertension, hyperkalemia, interstitial fibrosis, and progressive renal failure in transplant recipients. The cellular mechanisms, responding to these complications, were revealed in recent studies. CsA decreased the expression *iNOS* and production of the nitric oxide (NO) in mouse medullary thick ascending limbs (mTAL) cells. The alteration might subsequently affect the renal medullary hemodynamics and play a role in development of CsA nephrotoxicity. CsA decreased basolateral  $\text{Na}^+\text{-K}^+$  ATPase and increased apical  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  co-transport activity. The effects might subsequently account for the CsA-associated sodium retention, and decreased NO production. Decreased  $\text{Na}^+\text{-K}^+$  ATPase activity and enhanced  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  co-transport activity were the presentations of renal cell de-differentiation and proliferation. CsA increased mTAL cell proliferation by 2-fold and suggested the proliferation effect of CsA on renal epithelial cells. Activation of the renin-angiotensin system (RAS) is associated with renal fibrosis and progression of the renal failure. CsA enhanced intrarenal RAS activity mainly through the activation of the  $\text{AT}_1$  receptor by increasing the receptor numbers. The results suggest the role of the  $\text{AT}_1$  receptor antagonist in treating CsA nephrotoxicity. CsA also decreased the inflammation related intrarenal prostaglandin production via COX-2 production. Taken together, CsA altered cell proliferation, ionic transport, NO production, RAS and prostaglandins production in renal epithelial cells. The alterations were correlative and interactive to each other. The comprehension of the effect of CsA in renal epithelial cells gives us more insight in understanding drug-induced renal tubulointerstitial disease. (*Chang Gung Med J* 2007;30:7-16)

**Key words:** cyclosporine, nitric oxide, ion transport, renin-angiotensin system, renal epithelial cells

### Potent immunosuppressive agent: cyclosporine

Cyclosporine (CsA) selectively inhibits transcription of interleukin-2 (IL-2) and several other cytokines, mainly in T-helper lymphocytes.<sup>(1)</sup> The introduction of CsA in the 1980s significantly improved the survival of transplanted organs.<sup>(2)</sup> CsA is also becoming increasingly popular in the therapy

of various immune-mediated diseases.<sup>(3)</sup> However, concerns about long-term nephrotoxicity have restricted the use of these drugs to patients who have not responded to conventional treatment.<sup>(4,5)</sup> *In vivo*<sup>(6)</sup> and *in vitro*<sup>(7-16)</sup> research on CsA greatly enhanced our understanding of the drug nephrotoxicity during the last 10 years. The knowledge of the effects of CsA

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on the kidney also gives us an insight of the nature of the progression of renal failure from chronic tubulointerstitial disease, which constitutes a major cause of end-stage renal disease in Taiwan.<sup>(17)</sup>

### Cyclosporine nephrotoxicity

Patients treated with CsA are at high risk of developing renal injury.<sup>(18)</sup> CsA nephrotoxicity can manifest as acute azotemia, which is largely reversible after reducing the dose, or as irreversible chronic progressive renal disease.<sup>(19)</sup> Other renal effects of CsA include tubular dysfunction, and rarely a hemolytic uremic syndrome that can lead to acute graft loss.<sup>(19)</sup>

In the earliest clinical renal transplant trials using CsA, a high incidence of oliguric acute tubular necrosis was observed.<sup>(20)</sup> Animal studies have demonstrated that CsA causes vasoconstriction of the afferent and efferent arterioles and glomerular filtration rate.<sup>(21)</sup> The exact mechanism of the vasoconstriction is unclear. The increase in vascular resistance may be reflected clinically by an elevated plasma creatinine concentration and hypertension.<sup>(22)</sup> Acute CsA nephrotoxicity is usually reversible with cessation of therapy.<sup>(23)</sup>

Chronic CsA nephrotoxicity manifests as renal insufficiency, tubular function dysfunction, and an increase in blood pressure.<sup>(19)</sup> A cohort study of more than 11000 non-renal transplant recipients revealed that 17% of patients using CsA developed chronic renal failure (defined as an estimated GFR 29 mL/min per 1.73 m<sup>2</sup>) at a median follow-up of 36 months.<sup>(24)</sup> The risk continued to increase over time up to 5 years. These patients had a 4.6-fold increase in the risk of death compared with those without chronic renal failure. The mechanisms responsible for chronic CsA nephrotoxicity are not well understood. Renal failure progressed and reached end-stage renal failure in some of the patients. The understanding of the CsA renal effects is not only a research interest, but it is also clinically important in developing a strategy in the prevention and treatment of the disease.

### Cyclosporine in intrarenal hemodynamics

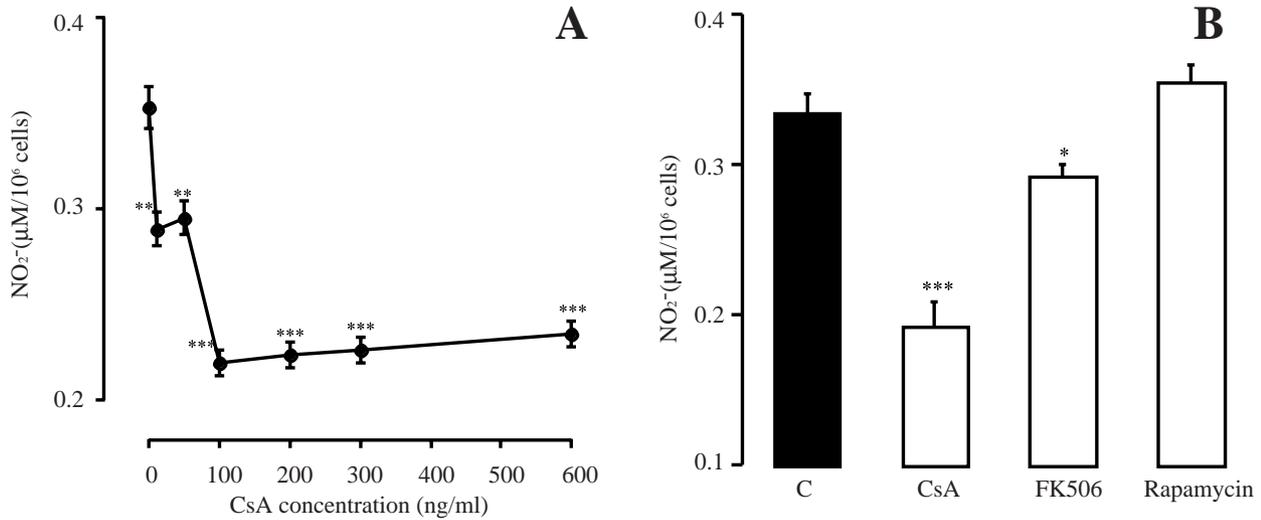
Nitric oxide (NO) plays an important role in the regulation of intra-renal vascular tone.<sup>(25)</sup> Its production is mediated by the activity of NO synthase (NOS). Tubular epithelial cells may constitutively

generate NO via iNOS.<sup>(26)</sup> *iNOS* mRNA has been shown to be mainly expressed in rat kidney medullary thick ascending limb cells and medullary collecting ducts without any NO inducer.<sup>(27)</sup> These results demonstrate constitutive expression of *iNOS* mRNA in rat medulla and suggest that NO may play a role in the hemodynamic regulation in this part of the kidney.<sup>(28)</sup>

CsA is nephrotoxic and has been responsible for progressive renal failure in some transplant patients.<sup>(6)</sup> CsA has been shown to enhance renal arterial vasoconstriction and decrease renal blood flow,<sup>(29)</sup> in part by altering the balance between vasodilating and vasoconstricting mediators, such as endothelin and NO/EDRF.<sup>(30)</sup> CsA stimulates the production of endothelin in endothelin cells.<sup>(30)</sup> It would be interesting to determine whether CsA has a direct effect on renal epithelial cells. Study results show that mTAL cells are the main site of steady *iNOS* gene expression in the kidney.<sup>(27)</sup> We investigated the effects of CsA on the production of NO in a model of sub-cultured mouse mTAL cells, which kept the specific functions of the parent cells from which they were derived.<sup>(8)</sup> We found that sub-cultured mTAL cells produced NO under basal conditions. Thus, NO produced by mTAL cells may act at the level of peritubular vessels to modulate blood flow and oxygenation. On the other hand, the mTAL sub-cultured cell represents an appropriate model for the study of intra-renal NO production. Our results showed that the CsA reduced the NO production via iNOS and reached its maximal effect at a rather low concentration of CsA (100 ng/ml), but not the other immunosuppressants (Fig. 1).<sup>(11)</sup> These results, in keeping with the association of CsA and clinical interstitial fibrosis, suggest that the inhibition of NO by CsA might interfere with the renal medullary vascular tone.

### Cyclosporine and ionic transport mechanisms

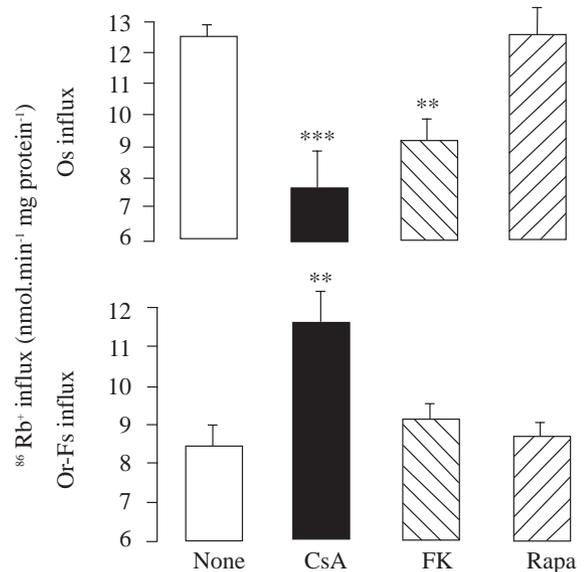
Determining how CsA affects the intra-renal production of NO is of interest. There have been increasing numbers of studies indicating that NO plays a role in the regulation of ionic transport within tubule epithelial cells. Guzman et al. had shown that the production of NO induced by LPS/IFN- $\gamma$  treatment inhibited the Na<sup>+</sup>-K<sup>+</sup> ATPase activity and reduced Na-dependent solute transport in a model of rat proximal tubule culture cells.<sup>(31)</sup> On the other



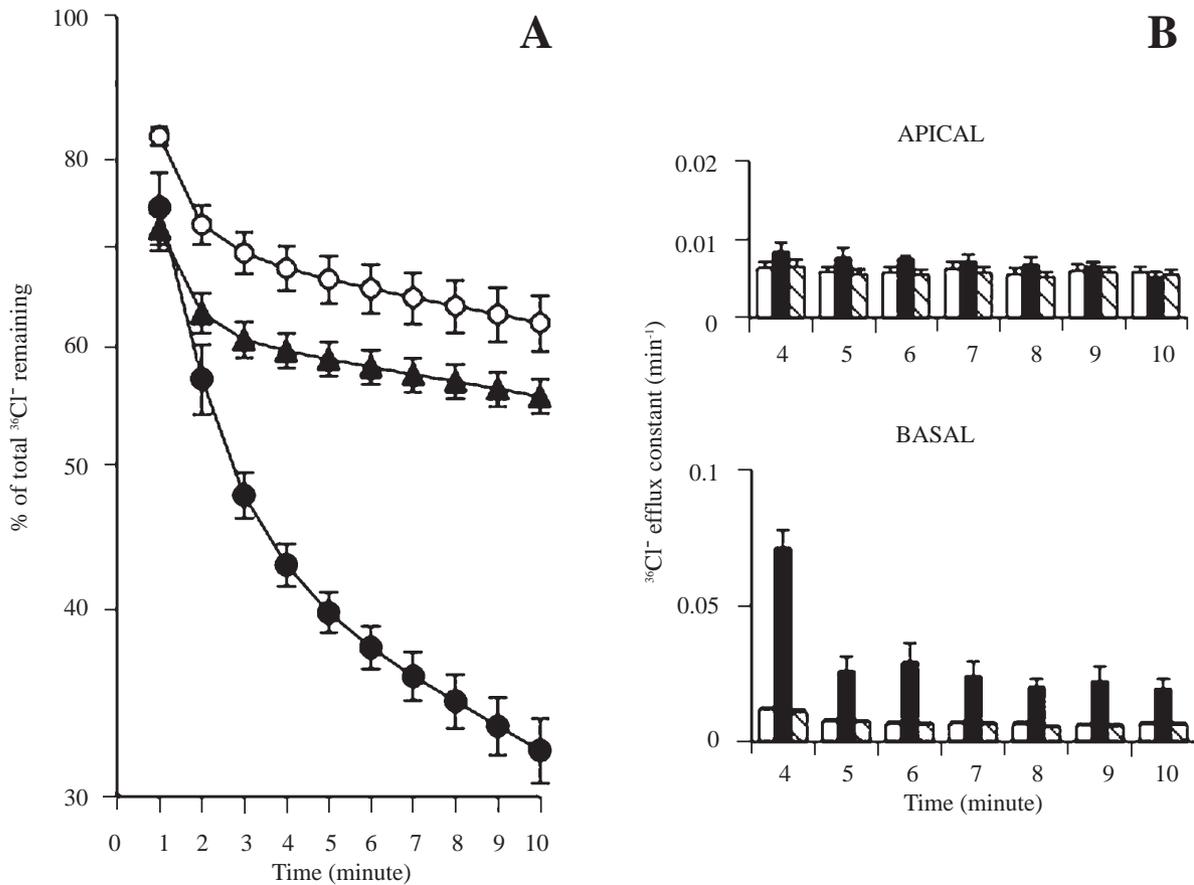
**Fig. 1** Effects of CsA, FK506 and rapamycin on the production of NO in mouse mTAL cultured cells. A: The release of NO<sub>2</sub><sup>-</sup> was measured on sets of confluent mTAL cells incubated without or with increasing concentrations of CsA for 6 hours at 37°C. B: The release of NO<sub>2</sub><sup>-</sup> was also measured on sets of confluent cells without (C, dark column) or with (open column) CsA (100 ng/ml), FK506 (10<sup>-7</sup> M) or rapamycin (10<sup>-7</sup> M) for 6 hours at 37°C. Values are means ± SE from 10-12 separate experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs control (C) values.

hand, Wu et al. showed that furosemide (10<sup>-5</sup> M) significantly enhanced the production of NO by 1.6-fold on cultured mTAL cells.<sup>(14)</sup> These results suggest that NO production in mTAL cells is associated with ion transport activity, directly or indirectly. Results of *in vivo* and *in vitro* studies have provided evidence that CsA exerts a direct inhibitory action on the renal Na<sup>+</sup>-K<sup>+</sup> ATPase pump<sup>(32-34)</sup> and impairs Na<sup>+</sup> absorption at the level of the proximal tubule cells,<sup>(35)</sup> mTAL cells<sup>(34)</sup> and distal tubule cells.<sup>(36)</sup>

The decrease in Na<sup>+</sup>-K<sup>+</sup> ATPase activity caused by CsA, especially in mTAL and collecting tubule cells, is thought to be one of the underlying mechanisms for the observed potassium and hydrogen ion secretion defects.<sup>(34)</sup> The NaCl absorption across TAL cells involves the apical Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> electroneutral co-transport, ATP-regulated potassium channels, basolaterally located Cl<sup>-</sup> channels and Na<sup>+</sup>-K<sup>+</sup> ATPase pumps.<sup>(37,38)</sup> We therefore analyzed the effects of CsA on Cl<sup>-</sup> and K<sup>+</sup> transport mediated by the electroneutral Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transport in a model of cultured mouse epithelial cells derived from microdissected mTAL cells.<sup>(8,11)</sup> CsA inhibited Na<sup>+</sup>-K<sup>+</sup> ATPase pumps activity by 38%. CsA also increased Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transport activity by 38% (Fig. 2), and stimulated the basolateral efflux of Cl<sup>-</sup> from mTAL cells grown on filters (Fig. 3). Apical K<sup>+</sup> channel (ROMK)



**Fig. 2** Effect of CsA, FK506 and rapamycin on <sup>86</sup>Rb<sup>+</sup> influx. The ouabain-sensitive (Os), representing Na<sup>+</sup>-K<sup>+</sup> ATPase activity, and the ouabain-resistant furosemide-sensitive (Or-Fs), representing Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporter activity, components of <sup>86</sup>Rb<sup>+</sup> influx were measured on sets of confluent TAL cells grown on Petri dishes and incubated without (Control) or with 100 ng/ml CsA (CsA), 10<sup>-7</sup>M FK506 (FK) or 10<sup>-7</sup>M rapamycin (Rapa). Values are the mean ± SE of ten separate experiments performed in duplicate. \*\* *p* < 0.01, \*\*\* *p* < 0.001 versus Control values.



**Fig. 3** Kinetics of  $^{36}\text{Cl}^-$  efflux from mTAL cells grown on filters. Confluent cells were loaded with  $^{36}\text{Cl}^-$  as described in Methods. After rinsing, fresh unlabeled PBS-CaCl<sub>2</sub> medium was added to both apical and basal sides of the filters. The 1 ml intervals sample-and-replace technique allowed to measure the percentage of  $^{36}\text{Cl}^-$  remaining in cells (A) and the rate constant of  $^{36}\text{Cl}^-$  efflux from the apical side (B, upper panel) and basal side (B, lower panel) of the cell layers incubated in the absence (É, open bars) or presence of 100 ng/ml CsA (J, black bars) or CsA plus 10<sup>-4</sup>M NPPB added to the basal side of the cells (H, striped bars). Values are the mean  $\pm$  SE from eight to ten separate experiments.

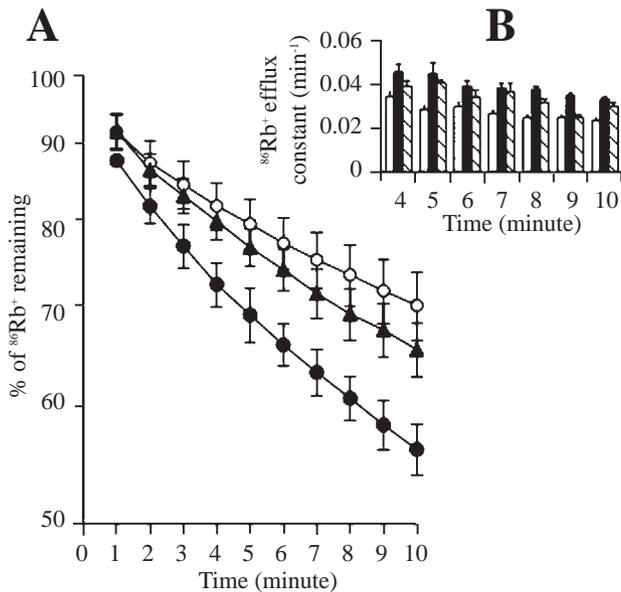
activity was greatly enhanced at the same time (Fig. 4). These results indicate that CsA may stimulate the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transport activity and suggest that CsA may interfere in the recycling of apical K<sup>+</sup> in this model of cultured mouse TAL cells.<sup>(10)</sup>

The transport process is regulated in a very narrow range by many intra-renal hormones.<sup>(39)</sup> The delicate transport processes keep our internal milieu in homeostasis. In addition, the ionic transport is a cellular signal in many cellular functions.<sup>(40)</sup> The ionic transport process participates in cell volume regulation,<sup>(41)</sup> apoptosis,<sup>(42)</sup> hormone excretion,<sup>(43)</sup> and many other cellular functions. The alteration of ionic transport might affect many cellular functions and lead to renal dysfunction acutely or chronically. We also

found that CsA increased the renal epithelial cells proliferation<sup>(15)</sup> which suggests a CsA effect on cell proliferation/differentiation in renal epithelial cells.

#### Cyclosporine and renin-angiotensin system

The renin-angiotensin system (RAS) is regulated by the ion transport and is involved in cell proliferation/differentiation in renal epithelial cells.<sup>(43)</sup> Activation of RAS also plays an important role in the process of CsA nephrotoxicity.<sup>(44)</sup> It has been shown that the inhibition of RAS by angiotensin converting enzyme (ACE) inhibitors or angiotensin II (Ang II) receptor antagonists can prevent the onset and progression of the renal toxicity caused by CsA.<sup>(45)</sup> In an animal study, CsA was reported to increase TGF- $\beta$

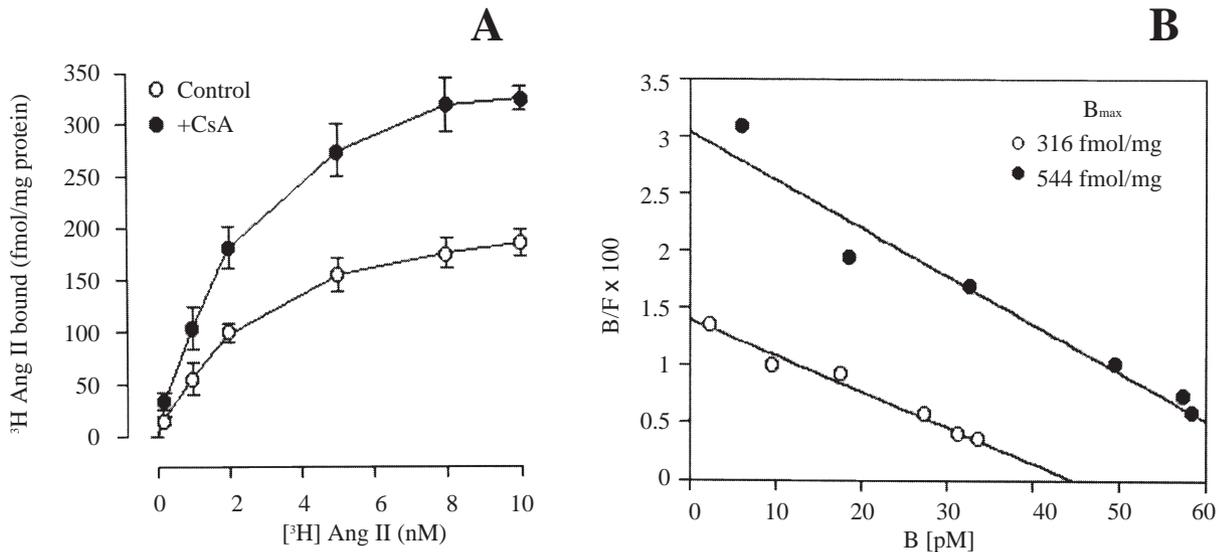


**Fig. 4** Kinetics of <sup>86</sup>Rb<sup>+</sup> efflux from TAL cells grown on Petri dishes. Confluent cells were loaded with <sup>86</sup>Rb<sup>+</sup>. After rinsing, fresh unlabeled PBS-CaCl<sub>2</sub> medium was added to the dish and the percentage of <sup>86</sup>Rb<sup>+</sup> remaining in cells (A) and the rate constant of <sup>86</sup>Rb<sup>+</sup> efflux (B) were measured on cells incubated in the absence (É, open bars) or presence of 100 ng/ml CsA (J, black bars) or CsA plus 10<sup>-4</sup>M Ba<sup>2+</sup> (H, stripped bars). Values are the mean ± SE from eight separate experiments.

“production, which is thought to be the common pathway of renal fibrosis.<sup>(46)</sup> The RAS blockade decreased the CsA-associated TGF-β production. The intrarenal Ang II content per gram of tissue is five-times greater in the medulla than the cortex of the rat kidney.<sup>(46)</sup> We investigated the effects of CsA on Ang II activity in sub-cultured mouse mTAL cells, which retained the main characteristics of the parental line from which they were derived.<sup>(8)</sup> CsA stimulated the number of AT<sub>1</sub> Ang II receptors (Fig. 5) and increased the intracellular level of calcium ([Ca<sup>2+</sup>]<sub>i</sub>), a downstream intracellular signal of Ang II,<sup>(47)</sup> measured either in the basal state or after Ang II stimulation.<sup>(12)</sup> The data indicated that CsA could stimulate Ang II activity in renal epithelial cells, which in turn suggests that it may be involved in CsA nephrotoxicity and progressive renal failure in recipients of kidney transplants.

#### Cyclosporine and prostaglandin

Prostaglandins (PGs) are important mediators in human physiology and disease. The PGs are derived from the metabolism of arachidonic acid by cyclooxygenase (COX).<sup>(48)</sup> The renal PGs have important local functions but are involved in only a few systemic activities, since they are rapidly metabolized in the



**Fig. 5** Effect of CsA on <sup>3</sup>H-Ang II binding in mTAL cells. A: Untreated (open circle) and CsA-treated (close circle) mTAL cells were incubated with various concentrations (0.2 to 10 nM) of <sup>3</sup>H-Ang II for 2h at 22°C. Points are the mean ± SD from 10 separate experiments. B: Scatchard plots of <sup>3</sup>H-Ang II binding to untreated (open circle) and CsA-treated (black circle) mTAL cells. Each point is the mean of 10 determinations performed in duplicate.

renal circulation.<sup>(48)</sup> The renal PGs are involved in three general areas of renal function. They play roles in the control of renin secretion, regulation of vascular tone, and control of tubular transport function.<sup>(49)</sup> It is in settings of compromised renal status that PGs can exert these functions to maintain renal blood flow and glomerular filtration rate.<sup>(50)</sup>

Different cell types along the nephron can synthesize PGE<sub>2</sub>.<sup>(51)</sup> Glomerular cells including epithelial, endothelial or mesangial cell are capable of generating PGE<sub>2</sub> as well as PGF<sub>2</sub> and PGI<sub>2</sub>.<sup>(52)</sup> Renal tubule cells, particularly those of the renal medulla, are important sites of renal PGs synthesis and PGE<sub>2</sub> is the major prostanoid synthesized.<sup>(51)</sup>

COX enzymes, both in human and animals, can be separated into two isoforms, COX-1 and COX-2. COX-1 is constitutively present in the renal vascular, glomerular cells and along most segments of tubules, although at different concentrations.<sup>(51)</sup> Basal levels of COX-2 are present in the macula densa, thick ascending limbs, and papillary interstitial cells under normal condition. COX-2 expression is markedly increased in volume-depleted rats and dogs.<sup>(53)</sup> The subsequent increase of PGE<sub>2</sub> mediates the local renal vascular dilatation and prevents ischemic renal injury.<sup>(53)</sup> On the other hand, the production of prostaglandins in renal epithelial cells is closely related with the ionic transport<sup>(54)</sup> and intrarenal RAS.<sup>(55,56)</sup>

Taken together, the effects of CsA on the intrarenal PGE<sub>2</sub> production, which plays a vital role in the regulation of the intra-renal hemodynamics, is important to understand.<sup>(53)</sup> We hypothesized that CsA can decrease *COX-2* formation with the consequence of decreases in PGE<sub>2</sub> formation via the inhibition of NO production in renal TAL cells, which are the main target of CsA tubular effects and play an important role in the regulation of intrarenal hemodynamics.

CsA reduced *COX-2* expression in lipopolysaccharide (LPS) stimulated TAL cultured cells, but not during the basal state in RT-PCR studies. Taking TAL cells without LPS as the control, 100 ng/ml LPS alone increased *COX-2* mRNA by  $3.97 \pm 0.30$  fold. LPS induced *COX-2* mRNA can be blocked by CsA. *COX-1* expression was not affected by CsA in either basal nor LPS stimulated conditions. Similar results were seen in other RT-PCR studies. The stimulated *COX-2* expression was inhibited by CsA in

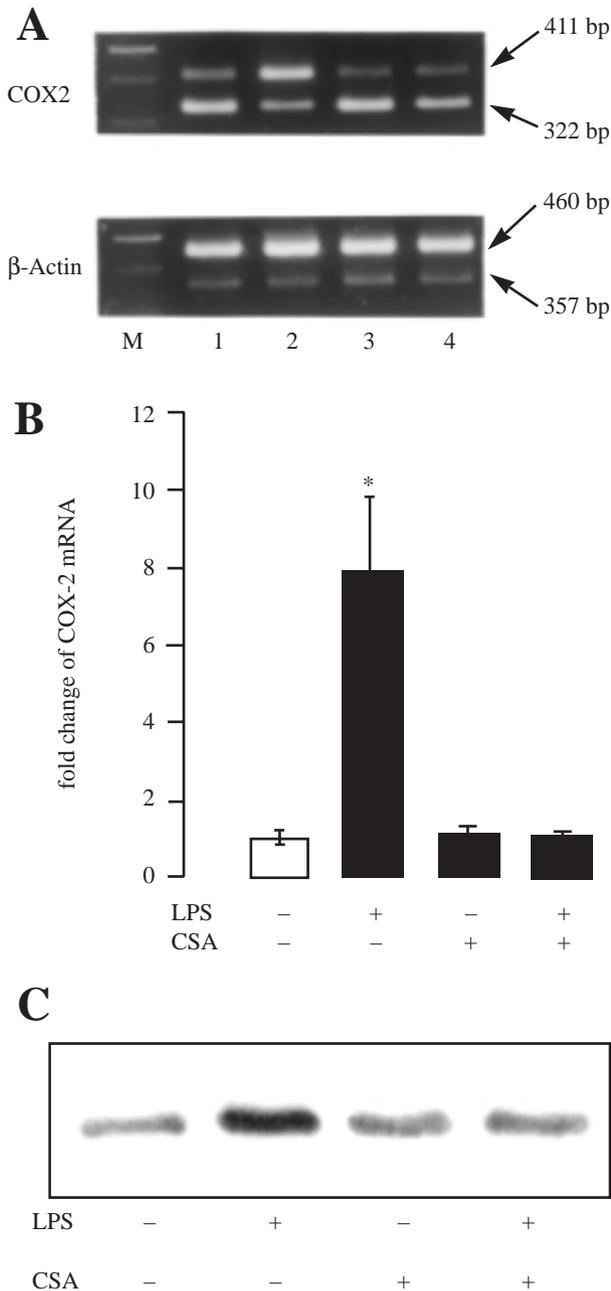
TAL cultured cells. Both the results of the RT-PCR and other RT-PCR studies suggest that CsA might decrease *COX-2* expression in the LPS-stimulated TAL cultured cell model. To further confirm this conclusion, we did Western blotting for cells cultured under similar conditions as in that of RT-PCR. It was shown that LPS enhanced *COX-2* protein expression and CsA reversed this enhancement (Fig. 6).<sup>(16)</sup>

Parallel to the *COX-2* RNA expression change, LPS induced a significant increase in PGE<sub>2</sub> production. CsA (100 ng/ml) significantly reduced PGE<sub>2</sub> production up to 50.1% in LPS stimulated TAL cells. CsA did not alter the basal PGE<sub>2</sub> production. The effect of CsA in significantly preventing LPS induced PGE<sub>2</sub> increase was dose-dependent from 10 ng/ml up to the concentration of 600 ng/ml.

NO plays a role in the regulation of PGE<sub>2</sub> production by regulating *COX-2* activity. It would be interesting to determine whether CsA associated PGE<sub>2</sub> decrease is associated with NO. We co-incubated LPS stimulated TAL cultured cells with CsA and SNAP (10<sup>-5</sup>M) (a nitric oxide donor).<sup>(11)</sup> PGE<sub>2</sub> production was enhanced in SNAP-treated TAL cells with or without LPS. CsA suppressed PGE<sub>2</sub> production in LPS stimulated TAL cultured cells was reversed by SNAP. CsA also decreased the NO production in LPS-stimulated TAL cells. The results indicate that NO might play an important role in the CsA-mediated PGE<sub>2</sub> decrease in TAL cultured cells.

#### Effect of cyclosporine in renal epithelial cells

The use of CsA greatly improves the graft survival in transplant allografts. The appearance of the CsA nephrotoxicity is a major drawback of the immunosuppressant. CsA exerted its effect via altering the ionic transport activity,<sup>(10)</sup> which subsequently led to cell proliferation,<sup>(15)</sup> activation of RAS,<sup>(12)</sup> inhibition of NO secretion<sup>(11)</sup> and PGE<sub>2</sub> production,<sup>(16)</sup> which possibly led to renal failure. The sequence of the study provided us an example of how a simple drug might affect the renal function in many facets leading to renal damage. The results of the bench studies suggest that calcineurin inhibitors, including CsA, could be minimized or avoided to prevent possible nephrotoxicity. The progressive nature of CsA nephrotoxicity also presented an example of the progression of renal failure from renal tubulointerstitial disease, which was an important component in the



**Fig. 6** (A) Representative RT-competitive PCR gel and the results of *COX-2* mRNA by CsA (M represented molecular weight standards and the other numbers represented the same manipulation as in Fig 4). (B) Bar chart showed that the LPS (100 ng/ml) induced a 8-fold ( $7.92 \pm 1.92$ ) ( $*p < 0.001$ ,  $n = 4$ ) increase of *COX-2* expression. CsA prevented the stimulatory effect of LPS. (C) Representative western blotting of *COX-2* protein showed that LPS can enhance *COX-2* protein expression and this enhancement can be blocked by CsA.

development of end-stage renal disease, especially in Taiwan. Abuse of herbal medications and other medications are common in Taiwan. Drug-related renal tubulointerstitial disease is another major area of study we should focus on.

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# 從病房到實驗室藥物引起之腎間質疾病 —培養腎上皮細胞之研究模式

吳麥斯

環孢靈是一種強力的免疫抑制劑，用於許多器官移植患者。環孢靈常合併有鉀離子留滯、高血壓、高血鉀症、腎間質纖維化和漸進性之腎衰竭。造成這些合併症之機轉，現在逐漸被研究清楚。在腎髓質亨利氏小管厚上行肢細胞中，環孢靈下降了；NOS 之表現，減少 NO 之產生，這樣的變化可能造成了腎臟髓質區域血液動力學之變化，進而在環孢靈腎毒性扮演一角色。環孢靈下降了，腎上皮細胞基底膜之  $\text{Na}^+ - \text{K}^+ \text{ATPase}$ ，上升上層膜之  $\text{Na}^+ - \text{K}^+ - \text{Cl}^-$  共同轉導器，這樣的作用解釋了環孢靈所引發之鈉滯留和 NO 產生下降，這樣轉導器活性改變更導致細胞體積之改變而影響細胞之生長與分化，我們先前之研究也顯示出環孢靈造成腎上皮細胞之增生高達兩倍。腎血管張素之活化和腎纖維化及腎衰竭之進展有很大的關連。環孢靈經過增加  $\text{AT}_1$  接受器之密度，增加了腎內腎素血管張素系統之活化。這樣的發現也顯示了  $\text{AT}_1$  接受器之阻斷可能可以改善環孢靈腎毒性。我們更進一步發現環孢靈可能下降腎內前列腺素之產生，進而改變了腎內之血液動力學和炎症狀態。總括而言，環孢靈改變腎上皮細胞之增生，離子傳導特性，NO 之產生，腎血管張素系統之活化和腎內前列腺素之產生。這些因素加上許多新的發現都是互相影響。對於環孢靈之研究，讓我們對藥物所引發之腎小管間質疾病有更深一層之了解，進而對於如何保護腎小管間質疾病有更多的研究和進展。(長庚醫誌 2007;30:7-16)

**關鍵詞：**環孢靈，一氧化氮，離子傳導，腎素血管張素系統，腎上皮細胞。

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