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Individual or Combined Effects of Enalapril and Verapamil on Chronic Cyclosporine Nephrotoxicity in Rats

Previous studies have demonstrated that enalapril and verapamil seem to attenuate the cyclosporine nephrotoxicity. However, the mechanisms have not been completely understood, especially on molecular events. The aim of this study was to examine the effect of individual or combined treatment on osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) mRNA expressions. Enalapril (50 mg/L in drinking water) and verapamil (0.5 mg/kg/day, subcutaneously), alone or in combination, were administered to rats with chronic cyclosporine nephrotoxicity (cyclosporine, 25 mg/kg/day, subcutaneously) (n=5 each). Five rats treated with olive oil vehicle were used as control. After 4 weeks, biochemical parameters were measured, and renal cortical mRNA levels were evaluated by Northern blot analysis. Cyclosporine reduced renal creatinine clearance significantly and induced renal cortical osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) gene expressions around 13.5$\pm$1.3, 2.4$\pm$0.2, 1.5$\pm$0.1, 1.9$\pm$0.1 folds, respectively. Individual treatment with enalapril or verapamil significantly suppressed the osteopontin and TGF-β mRNA expression, but not endothelin-1 and procollagen alpha 1(I). Combined treatment also inhibited the osteopontin and TGF-β mRNA expression but there was no difference between combined and individual treatment. In conclusion, enalapril or verapamil significantly blunted the cyclosporine-induced osteopontin and TGF-β gene expressions. However, combined treatment did not show any additive effect.

Key Words: *Cyclosporine; Enalapril; Gene expression; Verapamil*

Sang Koo Lee, Joo Yeol Park†, Eun Sil Yu*, Won Seok Yang, Soon Bae Kim, Su Kil Park, Jung Sik Park

Departments of Internal Medicine, Pathology* and Asan Institute for Life Sciences†, University of Ulsan, Seoul, Korea

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Address for correspondence
Sang Koo Lee, M.D.
Division of Nephrology, Department of Internal Medicine, Asan Medical Center, University of Ulsan, 388-1, Poongnap-dong, Songpa-gu, Seoul 138-736, Korea
Tel: +82.2-2224-3265, Fax: +82.2-2224-6963

INTRODUCTION

Cyclosporine is one of the most important immunosuppressive agents in renal transplantation. Despite its great benefit, cyclosporine may be associated with nephrotoxicity, which is either acute hemodynamic or chronic progressive. Acute cyclosporine nephrotoxicity seems to be induced by cyclosporine-induced vasoconstriction, which is mediated by endothelin, thromboxane, reduction of vasodilatory prostaglandins, reduced nitric oxide production and activation of renal sympathetic nervous systems (1). However, the mechanisms underlying chronic cyclosporine nephrotoxicity are not fully understood. Angiotensin II and angiotensin-dependent upregulation of molecules that are important in the scarring process, such as TGF-β and osteopontin, have been suggested (2-4). Early increase in renal cortical procollagen alpha 1(I) mRNA level was also reported in chronic cyclosporine nephrotoxicity (5).

According to these suggested mechanisms, calcium channel blockers which can inhibit calcium-mediated vasoconstriction in vascular smooth muscle cell, and angiotensin converting enzyme inhibitors (ACE inhibitor) which can inhibit the activation of renin-angiotensin system, have been suggested as possible means of preventing cyclosporine nephrotoxicity (1, 2, 4). However, the effect of individual or combined treatment with calcium channel blocker and ACE inhibitor on these suggested mediators are not known well.

The aim of this study was to evaluate the effect of individual or combined treatment with enalapril and verapamil on renal cortical osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) gene expressions, which are implicated in the pathogenesis of cyclosporine nephrotoxicity.

MATERIALS AND METHODS

Animals and experimental design

Chronic cyclosporine nephrotoxicity was induced in rats as previously described (5, 6). Male Wistar rats (160-220 g) were randomly assigned to five groups of 5 animals each. Group 1 (Normal group) received olive oil vehicles subcutaneously only. Group 2 (CsA group) received cyclosporine dissolved in olive oil, 25 mg/kg/day, subcutaneously. Group 3 (E group) received cyclosporine and enalapril, 50 mg/L in drinking water. Group 4 (V group) received cyclosporine and verapamil, 0.5 mg/kg/day, subcutaneously. Group 5 (EV group) received cyclosporine, enalapril and verapamil. All rats were given free access to standard rat chow and water. Rats were sacrificed at 4 weeks after drug administration.

The following measurements were made immediately prior to sacrifice: body weight, left kidney weight, serum and 24-hr urinary creatinine. Systolic arterial pressure was measured by the tail cuff method. After sacrifice, the left kidney was removed, and processed. For RNA preparation, approximately 100 mg of renal cortical tissue was transferred to guanidinium thiocyanate buffer (Tri reagent kit®, Molecular Research Center, INC, Cincinnati, OH, U.S.A.), frozen in liquid nitrogen immediately and stored at -80℃ until processing. For light microscopic analysis, renal cortical tissue was fixed in 10% neutral buffered formalin.

RNA isolation and Northern hybridization

Total cellular RNA was extracted using a commercially available modification of the acid-phenol method (Tri reagent kit®). Amount of RNA was quantitated by absorbance at 260 nm and purity of RNA was assessed by absorbance ratio at 260/280 nm. Northern hybridization was performed as previously described (7). Briefly, total RNA (15 μg/lane) was electrophoresed through 1% agarose, 2.2 M formaldehyde denaturing gel with MOPS buffer, followed by capillary transfer to nylon filters. Filters were stained with methylene blue, to check the integrity, uniformity of loading and transfer of RNA, prior to fixation in vacuo at 80℃ for 2 hr. Filters were prehybridized at 65℃ in 0.5 M $NaHPO_4$ buffer, pH 7.0, 1 mM EDTA, 7% sodium dodecyl sulfate and 1% bovine serum albumin for 4 to 6 hr. Then filters were hybridized with ^{32}P labeled cDNA probes and 100 μg/mL salmon sperm DNA at 65℃ overnight. cDNA probes were labeled with ^{32}P using random primers (Megaprime DNA labeling system, Amersham International plc, England). Filters were washed and exposed to film at -80℃. Intensities of the bands on autoradiogram were quantitated by scanning laser densitometry (GS-670 imaging densitomter, Bio-Rad). The same filters were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) to correct for variation in RNA loading and transfer efficiency.

Generation of cDNA probes

cDNA probe for rat TGF-β was obtained from ATCC (American Type Culture Collection). Other cDNA probes for rat osteopontin, endothelin-1, procollagen alpha I (I), and GAPDH were made by reverse transcription-polymerase chain reaction (RT-PCR) method. RT-PCR was performed as previously described (7). Briefly, oligo (dT) (0.5 μg, Boehringer-Mannheim, Indianapolis, IN, U.S.A.) was annealed to total RNA (20 μg, from interleukin-1-stimulated rat mesangial cells or rat spleen) after denaturation at 70℃ and cDNA was synthesized at 42℃ for 1 hr using 100 U of Superscript (BRL, Gaithersburg, MD, U.S.A.) in a final volume of 20 μL of the vendor's buffer supplemented with 20 U RNAsin (Promega Biotec, Madison, WI, U.S.A.). cDNA was precipitated and dissolved in 1 mM EDTA, 10 mM Tris, pH 7.4. After RT, PCR was performed in PCR buffer containing 0.25 mM dNTP, 12.5 pmol of each primer and 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) in a total volume of 50 μL with initial denaturation at 94℃ for 3.5 min, followed by 30 cycles of 94℃ for 1.5 min, 54℃ for 1.5 min and 72℃ for 1.5 min. After 30 cycles, a final extension at 72℃ for 8 min was performed. Each PCR primer set was synthesized (from Korea Biotech, Inc, Korea) according to the published cDNA sequences for rat osteopontin (8), endothelin-1 (9), procollagen alpha I (I) (10) and GAPDH (11).

The sequences of each primer set were as follows:

For osteopontin,
forward primer 5' CCTCCTGTCTCCCGGTGAA 3'
backward primer 5' CTCGGCACTATCGATCGCA 3'

For endothelin-1,
forward primer 5' TCCGCTCGCTGCCTTCTCT 3'
backward primer 5' GCTTCAGACAGGCCCCGAA 3'

For procollagen alpha 1(I),
forward primer 5' GATGTGCCACTCTGACTGG 3'
backward primer 5' ACATCGATGATGGGCAGGC 3'

For GAPDH,
forward primer 5' TCACCATCTTCCAGGAGCG 3'
backward primer 5' CTGCTTCACCACCTTCTTGA 3'

PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel and the expected size of each PCR product was confirmed under ultraviolet illumination. PCR products were purified using a Jetsorb gel extraction kit (Genomed Inc, U.S.A.)

Table 1. Biochemical and functional parameters in each groups

	Normal	CsA group	E group	V group	EV group
Body weight (g)	332±10	276±18	296±11	290±10	256±13
Kidney weight/Body weight	0.43±0.02	0.39±0.02	0.40±0.02	0.41±0.02	0.43±0.03
Blood pressure (mmHg)	138±2	140±1	106±1*,†	137±2	105±3*,†
Serum creatinine (mg/dL)	0.58±0.02	0.68±0.04	0.66±0.03	0.62±0.02	0.70±0.01
Creatinine clearance (mL/min)	0.95±0.07	0.37±0.14*	0.43±0.13	0.43±0.18	0.50±0.13

Data are mean±SE, *p<0.05 vs. normal, †p<0.05 vs. CsA group
Normal group, rats treated with olive oil vehicle; CsA group, rats treated with cyclosporine; E group, rats treated with cyclosporine and enalapril; V group, rats treated with cyclosporine and verapamil; EV group, rats treated with cyclosporine, enalapril and verapamil

Statistical analysis

All data were expressed as mean±SE. Groups were compared using an analysis of variance for multiple group comparison (Scheffe's method). A value of p less than 0.05 was considered significant.

RESULTS

Biochemical and histologic changes

The mean values for body weight, kidney weight/body weight ratio, blood pressure, serum creatinine and creatinine clearance were listed in Table 1.

Creatinine clearance was significantly reduced in cyclosporine-treated rats, compared to normal rats. However, there were no differences in body weight, kidney weight/body weight ratio, blood pressure and serum creatinine between cyclosporine-treated rats and normal rats.

Treatment with enalapril significantly reduced blood pressure but verapamil had no effect on reducing blood pressure. There were no significant differences in body weight, kidney weight/body weight ratio, serum creatinine and creatinine clearance between cyclosporine-treated rats and enalapril- or verapamil-treated rats.

Light microscopic studies showed that administration of cyclosporine for 4 weeks induced scattered foci of tubular atrophy and small cytoplasmic vacuoles in the cortex. A few mononuclear cells were scattered in the interstitium but interstitial fibrosis was rarely observed. However, there were no significant differences in histologic changes between cyclosporine-treated rats and enalapril- or verapamil-treated rats.

Gene expressions of osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) after administration of cyclosporine

Administration of cyclosporine for 4 weeks increased the renal cortical osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) mRNA levels significantly around 13.5±1.3, 2.4±0.2, 1.5±0.1, 1.9±0.1 folds, respectively as compared with normal rats (Fig. 1-4). Osteopontin gene was more predominantly expressed than other genes.

The effect of individual or combined treatment with enalapril and verapamil on osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) gene expressions

Treatment with enalapril significantly suppressed the cyclosporine-induced osteopontin and TGF-β gene expressions down to 34.7±8.2%, 58.7±9.4%, respectively, but did not affect the endothelin-1 and procolla-

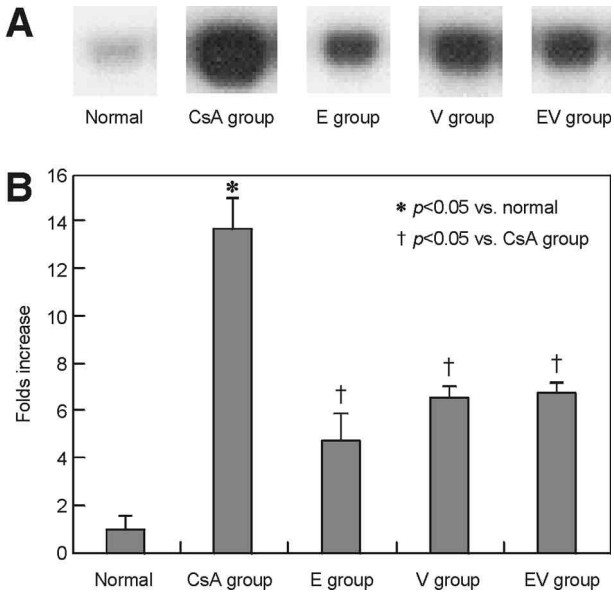

Fig. 1. Osteopontin mRNA expression. **A:** A representative of autoradiography. **B:** Densitometric quantitation of osteopontin mRNA: osteopontin mRNA level was normalized by GAPDH mRNA level. Normal group, rats treated with olive oil vehicle; CsA group, rats treated with cyclosporine (CsA); E group, rats treated with CsA and enalapril; V group, rats treated with CsA and verapamil; EV group, rats treated with CsA, enalapril and verapamil (n=5 each group).

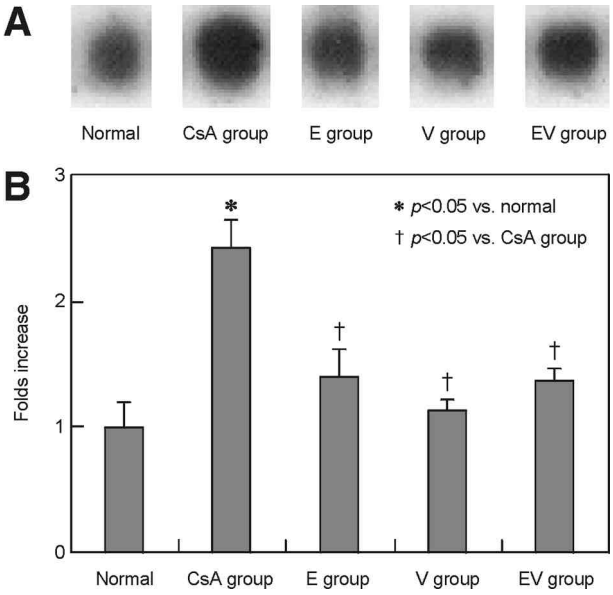

Fig. 2. TGF-β mRNA expression. **A:** A representative of autoradiography. **B:** Densitometric quantitation of TGF-β mRNA: TGF-β mRNA level was normalized by GAPDH mRNA level. Normal group, rats treated with olive oil vehicle; CsA group, rats treated with cyclosporine (CsA); E group, rats treated with CsA and enalapril; V group, rats treated with CsA and verapamil; EV group, rats treated with CsA, enalapril and verapamil (n=5 each group).

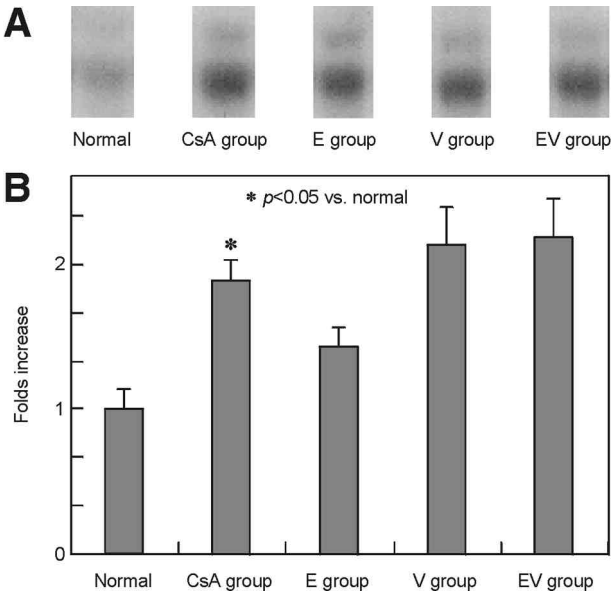

Fig. 3. Procollagen alpha 1(I) mRNA expression. **A:** A representative of autoradiography. **B:** Densitometric quantitation of procollagen alpha 1(I) mRNA: procollagen alpha 1(I) mRNA level was normalized by GAPDH mRNA level. Normal group, rats treated with olive oil vehicle; CsA group, rats treated with cyclosporine (CsA); E group, rats treated with CsA and enalapril; V group, rats treated with CsA and verapamil; EV group, rats treated with CsA, enalapril and verapamil (n=5 each group).

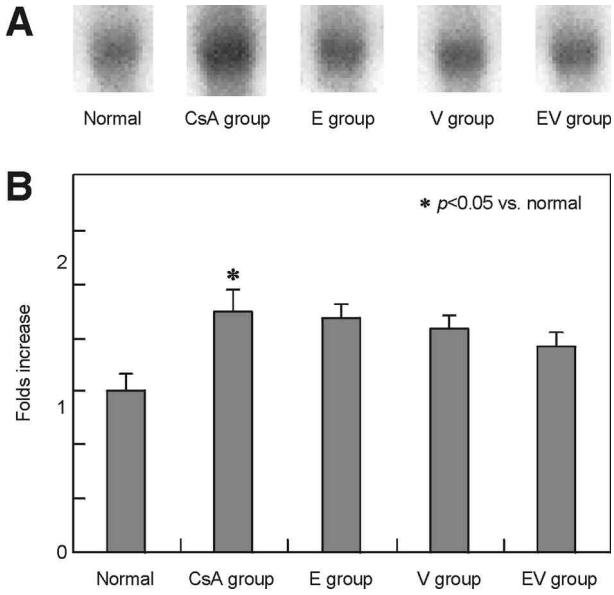

Fig. 4. Endothelin-1 mRNA expression. **A:** A representative of autoradiography. **B:** Densitometric quantitation of endothelin-1 mRNA: endothelin-1 mRNA level was normalized by GAPDH mRNA level. Normal group, rats treated with olive oil vehicle; CsA group, rats treated with cyclosporine (CsA); E group, rats treated with CsA and enalapril; V group, rats treated with CsA and verapamil; EV group, rats treated with CsA, enalapril and verapamil (n=5 each group).

gen alpha 1(I). Treatment with verapamil significantly suppressed the cyclosporine-induced osteopontin and TGF-β gene expressions down to 49.0±2.6%, 45.9±2.2%, respectively, but did not affect the endothelin-1 and procollagen alpha 1(I). Combined treatment with enalapril and verapamil also significantly suppressed cyclosporine-induced osteopontin and TGF-β gene expressions down to 49.7±1.7%, 57.4±1.1%, respectively, but there was no differences in osteopontin and TGF-β mRNA level between combined and individual treatment (Fig. 1-4).

DISCUSSION

Previous studies have suggested that enalapril or verapamil seem to attenuate the cyclosporine nephrotoxicity (1, 2, 4). But the effect of individual or combined treatment on the osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) gene expressions, which have been implicated in the pathogenesis of cyclosporine nephrotoxicity, are not known well. Our study showed that individual treatment with enalapril or verapamil significantly suppressed the cyclosporine-induced osteopontin and TGF-β gene expressions, but not endothelin-1 and procollagen alpha 1(I). However, combined treatment did

not show any additive effect.

Osteopontin is a highly acidic glycosylated phosphoprotein with Arg-Gly-Asp (RGD) sequences and its biological roles seem to be involved in facilitating cell attachment and spreading by interacting with vitronectin receptor. It also acts as a chemoattractant for macrophages as well as smooth muscle cells (12). In several rat models with renal injury, osteopontin is markedly induced and overexpressed by proximal tubules, both at the mRNA and protein level. Osteopontin is also known as an important mediator in tubulointerstitial injury in glomerulonephritis (12). Recently, Narita et al. suggested that osteopontin might play an important role in the process which promote initial renal injury to chronic progressive injury in anti-Thy 1.1 antibody rat model (13). Thus, osteopontin seems to act as a major player in the renal injury and/or repair cascade.

A dramatic increase in osteopontin mRNA and protein levels in rats with chronic cyclosporine nephrotoxicity has been reported, and the degree of osteopontin expression correlated with the numbers of macrophages in the tubulointerstitium and also with the severity of interstitial fibrosis (4). Our results were consistent with their study, showing that osteopontin gene was markedly induced by cyclosporine and the mRNA level of osteopontin was much higher than that of other genes such as TGF-β, endothelin-1 and procollagen alpha 1(I). The mechanisms by which cyclosporine induces the osteopontin gene expression, are largely unknown. However, it is known that osteopontin expression can be induced in tubular epithelial cells by angiotensin II and/or by ischemia (14, 15). Our results showed that both enalapril and verapamil could suppress the cyclosporine-induced osteopontin gene expression. Thus, considering that verapamil also had an effect on decreasing the osteopontin mRNA levels, osteopontin gene expression might be induced rather by cyclosporine-induced ischemia, not by direct effect of angiotensin II. In our study, cyclosporine-induced ischemia might be ameliorated either by angiotensin II- blockade effect of enalapril or by the vasodilatory effect of verapamil. There was also a report that angiotensin II did not appreciably alter the osteopontin gene expression in cultured rat renal epithelial cells (16). If this was the case, it could explain, at least in part, why combined treatment with enalapril and verapamil did not show any additive effect on suppressing the cyclosporine-induced osteopontin gene expressions in our study. Similar to our results, Pichler et al. had demonstrated that hydralazine, a vasodilatory drug, could reduce the osteopontin expressions in renal tissue of cyclosporine nephropathy (4).

TGF-β is a key fibrogenic cytokine implicated in the fibrosis of a number of chronic diseases of the kidney and other organs. An increase in TGF-β mRNA and protein levels in rats with chronic cyclosporine nephrotoxicity has been reported (3, 4, 6). Our results were in accordance with their study. The mechanism by which cyclosporine induces TGF-β gene expressions has not been fully understood, but direct effect of angiotensin II has been suggested (17). However, various other stimuli including renal ischemia would be able to induce the TGF-β gene expression (18). Our result which showed that verapamil as well as enalapril significantly suppressed the cyclosporine-induced TGF-β gene expression, suggested that cyclosporine-induced TGF-β gene expression was dependent not only on angiotensin II, but also on other stimuli such as renal ischemia. Similar to our results, there was a report that hydralazine, a vasodilator, could reduce the TGF-β expression in renal tissue of cyclosporine nephropathy (4). However, there has been a controversy over the effect of hydralazine on cyclosporine-induced TGF-β gene expression (17).

Early and sustained increase in renal cortical procollagen alpha 1(I) mRNA level in rats with chronic cyclosporine nephrotoxicity has been reported (2, 5, 17). Our results showed that cyclosporine induced significant elevation of cortical procollagen alpha I (I) mRNA level, which could not be attenuated by enalapril or verapamil. Our results were consistent with the study of Burdmann et al. (2). In contrast to our study, Shihab et al. reported that enalapril could reduce the procollagen alpha I (I) mRNA levels in renal tissue of cyclosporine-treated rats (17). This discrepancy might be related to the different dose and route of administration for enalapril, different kind of rats and different degree of activation of renin-angiotensin system in rats. In the study of Shihab et al., enalapril was administered at the dose of 10 mg/kg by garbage to Spraque-Dawley rats that received the low-salt diet (17). On the other hand, in our study, enalapril (50 mg/L in drinking water) was administered to Wistar rats that received the normal salt diet. It has been known that only salt-depleted animals developed significant interstitial fibrosis by cyclosporine through the activation of renin-angiotensin system (19). As a matter of fact, the procollagen alpha I (I) mRNA level of cyclosporine-treated rats in our study was much lower than that of Shihab et al. and also renal interstitial fibrosis was rarely observed in cyclosporine-treated rats of our study.

Endothelin is known to be involved mainly in acute cyclosporine nephrotoxicity (1). An increase in endothelin-1 mRNA level in renal medulla of rats treated with cyclosporine for a short period has been reported (20). Our results revealed that endothelin-1 mRNA level was increased also in the renal cortex of rats treated with cyclosporine and sustained for 4 weeks. Similar to our study, Takeda et al. reported that endothelin-1 mRNA level was increased in the mesenteric arteries of hyper-

tensive rats induced by cyclosporine for 6 weeks treatment (21). However, it is not clear how much endothelin-1 contributes to the pathogenesis of chronic cyclosporine nephrotoxicity. In our study, neither enalapril nor verapamil could suppress cyclosporine-induced endothelin-1 gene expressions.

In summary, administration of cyclosporine for 4 weeks in rats significantly reduced the renal creatinine clearance and induced the renal cortical osteopontin, TGF-β, endothelin-1 and procollagen alpha 1(I) gene expressions. Individual treatment with enalapril or verapamil significantly suppressed the cyclosporine-induced osteopontin and TGF-β gene expressions but not endothelin-1 and procollagen alpha 1(I). However, combined treatment did not show any additive effect.

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