

# Effect of calcium channel antagonist diltiazem and calcium ionophore A23187 on cyclosporine A-induced apoptosis of renal tubular cells

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**Abstract** Calcium channel antagonists have been reported to have a favorable impact on cyclosporin A (CsA)-treated kidney transplant recipients. However, it is not clear whether this is because of their direct effect on antagonizing the toxicity of CsA to renal tubular cells. In this study, we have used Madin–Darby canine kidney tubular cells as a model to examine the effect of diltiazem, a calcium channel antagonist, on CsA-induced apoptosis. Moreover, to investigate the possible regulation of CsA cytotoxicity by intracellular calcium level, the effect of the calcium ionophore A23187 on CsA-induced apoptosis was also examined. We found that treatment of CsA (20  $\mu$ M) alone caused 20–30% cell death, which was apparently (30–40%) enhanced by diltiazem at 100  $\mu$ g/ml, accompanied by more severe DNA fragmentation, activation of caspases, and a decreased level of Bcl-2. The caspase inhibitor ZVAD-fmk or Bcl-2 overexpression was capable of suppressing apoptosis induced by the synergistic effect of diltiazem and CsA. Moreover, the survival rate of cells treated with CsA (30  $\mu$ M) alone remained only 30%, however, it was markedly ( $\sim$ 40%) elevated by co-treatment with A23187 (75 ng/ml). The rescue of cells from CsA-induced apoptosis by A23187 was correlated with AKT activation, BAD phosphorylation, and caspase-3 inactivation. Taken together, our results suggest that the reported favorable impact of diltiazem on kidney grafts is likely not because of its direct protection on renal tubular cells. Instead, it enhances the toxicity of CsA to renal tubular cells. In addition, our findings raise a possibility that the intracellular calcium level and the AKT pathway may participate in the regulation of CsA cytotoxicity. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Cyclosporin; Apoptosis; Diltiazem; Calcium ionophore; AKT

## 1. Introduction

The immunosuppressive agent cyclosporin A (CsA) is widely used for the management of organ transplantation and in the treatment of various autoimmune disorders [1].

However, its adverse effect on renal structure and function makes nephrotoxicity a major limiting side effect [2]. Chronic CsA nephrotoxicity can progress to an irreversible lesion characterized by tubular atrophy, loss of tubular cells, and interstitial fibrosis [3,4]. The pathogenesis of chronic CsA nephrotoxicity has been thought to be secondary to the hemodynamic changes elicited by the intense effect of CsA on vasoconstriction [5]. However, increasing evidence also indicates that CsA has a direct toxicity to renal tubular cells, leading to their apoptosis [6]. An increased rate of tubular cell apoptosis was observed in human renal biopsy specimens obtained from patients with CsA nephrotoxicity [7]. In rats [8,9] or in vitro cell culture [10,11], CsA has been shown to induce tubular cell apoptosis, correlated with the activation of the caspase cysteine proteases and the altered expression of pro- or anti-apoptotic proteins, such as p53, the Bcl-2 family proteins, and Fas/Fas-L proteins.

From animal studies and few clinical reports [12–16], it appears that the calcium channel antagonists, such as diltiazem and verapamil, have a favorable impact on CsA-treated kidney transplant recipients. Although the detailed mechanism is not clear, one of the possibilities is that calcium channel antagonists may have a direct ‘cytoprotective’ effect on renal tubular cells. In this study, we set out to examine this possibility in Madin–Darby canine kidney (MDCK) cells.

## 2. Materials and methods

### 2.1. Chemicals

CsA was purchased from Novartis Pharmaceutical. Diltiazem HCl (Herbesser<sup>®</sup>) was purchased from Tanabe Seiyaku Co. (Osaka, Japan). Calcium ionophore A23187 was purchased from Calbiochem (San Diego, CA, USA). Caspase inhibitor ZVAD-fmk was purchased from Takara Shuzo Co. (Shiga, Japan). The monoclonal anti-Bcl-2 was purchased from Transduction Laboratories (Lexington, KY, USA). The rabbit polyclonal anti-AKT, anti-phosphoAKT (Ser473), and anti-cleaved caspase-3 and the monoclonal anti-phosphoBAD (Ser112) were purchased from New England Biolabs (Beverly, MA, USA).

### 2.2. Cell culture

MDCK cells were obtained from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air atmosphere. MDCK cells stably overexpressing Bcl-2 have been described previously [17]. Cells were seeded at  $3 \times 10^5$  per 60-mm culture dish in growth medium for 36 h before pharmacological treatments.

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**Abbreviations:** CsA, cyclosporin A; MDCK, Madin–Darby canine kidney

To examine the effect of diltiazem on CsA cytotoxicity, cells were treated simultaneously with 20  $\mu$ M CsA and various doses (mostly 100  $\mu$ g/ml) of diltiazem for 24 h. To examine the effect of A23187 on CsA cytotoxicity, cells were pretreated with various doses (mostly 75 ng/ml) of A23187 for 1 h before CsA addition to 30  $\mu$ M. Those cells were further incubated for 24 h before analysis. In some experiments, cells were pretreated with the caspase inhibitor ZVAD-fmk at 60  $\mu$ M for 1 h before CsA addition.

### 2.3. Measurements of cell survival, DNA fragmentation, and caspase activity

Cell viability was determined by trypan blue exclusion. Briefly, CsA-treated cells including both floating and adhesive cells were collected and washed once with phosphate-buffered saline (PBS). Cell suspensions in PBS were mixed with an equal volume of 0.4% trypan blue (Sigma) and cells excluding dye were counted with a hemocytometer under a light microscope. Cell survival was expressed as the percentage of live cells remaining after 24 h CsA treatment compared to the cell number at the time right before CsA addition.

To measure the extent of DNA fragmentation, cells were collected as described above and subjected to a colorimetric enzyme immunoassay for determination of histone-associated DNA fragments using a Cell Death Detection ELISA<sup>PLUS</sup> kit (Boehringer Mannheim) following the manufacturer's instructions. Each experiment was performed in duplicate.

The activities of caspase-1, 2, 3, 4, 8, 9, and 10 were measured by Caspase Colorimetric Protease Assay kits from BioVision Research Products (Palo Alto, CA, USA) following the manufacturer's instructions. Each experiment was performed in duplicate.

### 2.4. Immunoblotting

24 h after CsA treatment, cells were washed twice with PBS and lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, and 1 mM  $\text{Na}_3\text{VO}_4$ ) containing protease inhibitors as described previously [18]. The lysates were centrifuged for 10 min at 4°C to remove debris, and the protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA, USA). The lysates (50  $\mu$ g) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, USA). Immunoblotting was performed with appropriate antibodies using the NEN Life Science chemiluminescence system for detection.

### 2.5. Statistics

Statistical analyses were performed with a simple *t*-test. Differences were considered to be statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Enhancement of renal cytotoxicity of CsA by calcium channel antagonist diltiazem

To examine whether calcium channel antagonists have a protective effect on CsA-induced cell death, MDCK cells were treated with 20  $\mu$ M CsA in the presence or absence of the L-type calcium channel antagonist diltiazem for 24 h (Fig. 1A). CsA alone induced 20–30% cell death. In the combined treatment of CsA with lower concentrations (1 and 10  $\mu$ g/ml) of diltiazem, the rate of cell death remained constant. However, the rate of cell death was 15 and 30% increased when diltiazem was at 50 and 100  $\mu$ g/ml, respectively. It is noteworthy that diltiazem at 100  $\mu$ g/ml had no detectable toxicity to MDCK cells within 24 h. Moreover, in time-course experiments (Fig. 1B), we found that within 24 h, CsA in combination with 100  $\mu$ g/ml diltiazem constantly led to ~30% more cell death than CsA alone did. Together, although administration of diltiazem has been reported to benefit CsA-treated kidney transplant recipients [13–16], our results clearly demonstrate that the toxicity of CsA to renal tubular cells is enhanced by diltiazem *in vitro*.

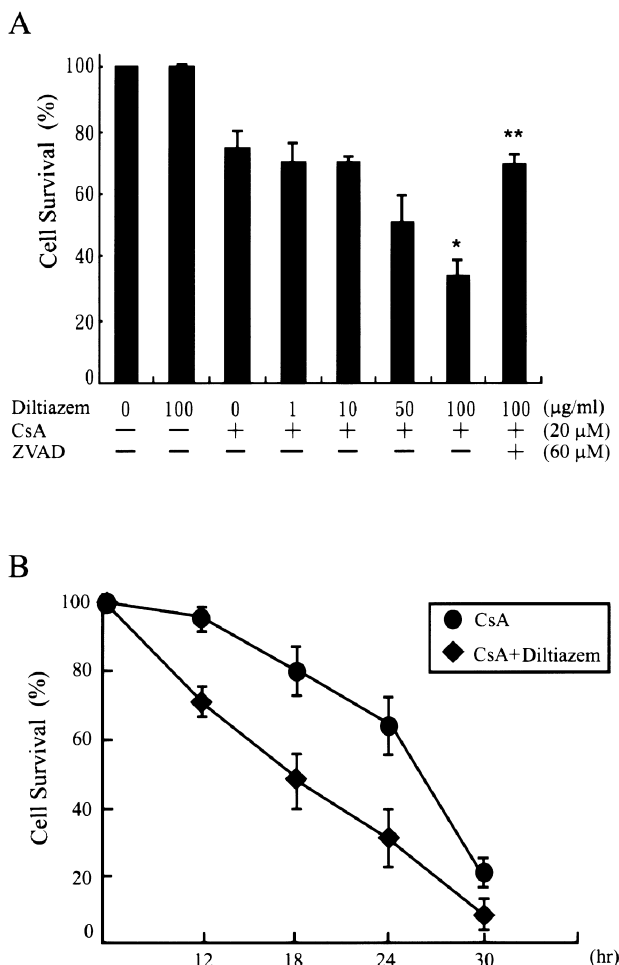


Fig. 1. Enhancement of renal cytotoxicity of CsA by calcium channel antagonist diltiazem. A: MDCK cells were treated with (+) or without (-) 20  $\mu$ M CsA in the presence of various concentrations of diltiazem as indicated. In one case, the broad range caspase inhibitor ZVAD-fmk (60  $\mu$ M) was added to the culture 1 h before CsA addition. 24 h after CsA addition, live cells were determined by trypan blue exclusion. Cell survival is expressed as the percentage of live cells remaining compared with the cell number at the time right before CsA addition. Data (means  $\pm$  S.E.M.) are from three independent experiments. \* $P < 0.001$  (statistically significant compared with CsA alone). \*\* $P < 0.001$  (statistically significant compared with combined treatment of CsA and 100  $\mu$ g/ml diltiazem). B: MDCK cells were treated with CsA (20  $\mu$ M) alone or in combination with diltiazem (100  $\mu$ g/ml). After various times as indicated, cell survival was determined as described in A. Data (means  $\pm$  S.E.M.) are from three independent experiments.

### 3.2. Diltiazem enhancement of CsA-induced cell death results from increased apoptosis

CsA has previously been shown to induce apoptosis via caspase-dependent pathway in renal tubular cells [9,10]. To examine if caspases are involved in diltiazem enhancement of CsA-induced cell death, a broad range caspase inhibitor, ZVAD-fmk, was applied in the experiments (Fig. 1A). ZVAD-fmk at 60  $\mu$ M significantly suppressed cell death induced by 20  $\mu$ M CsA and 100  $\mu$ g/ml diltiazem, suggesting a possible involvement of caspases in diltiazem-enhanced cell death. To further examine the possibility that diltiazem potentiates the effect of CsA on apoptosis, the extent of DNA fragmentation, a characteristic of apoptosis, was measured using a colorimet-

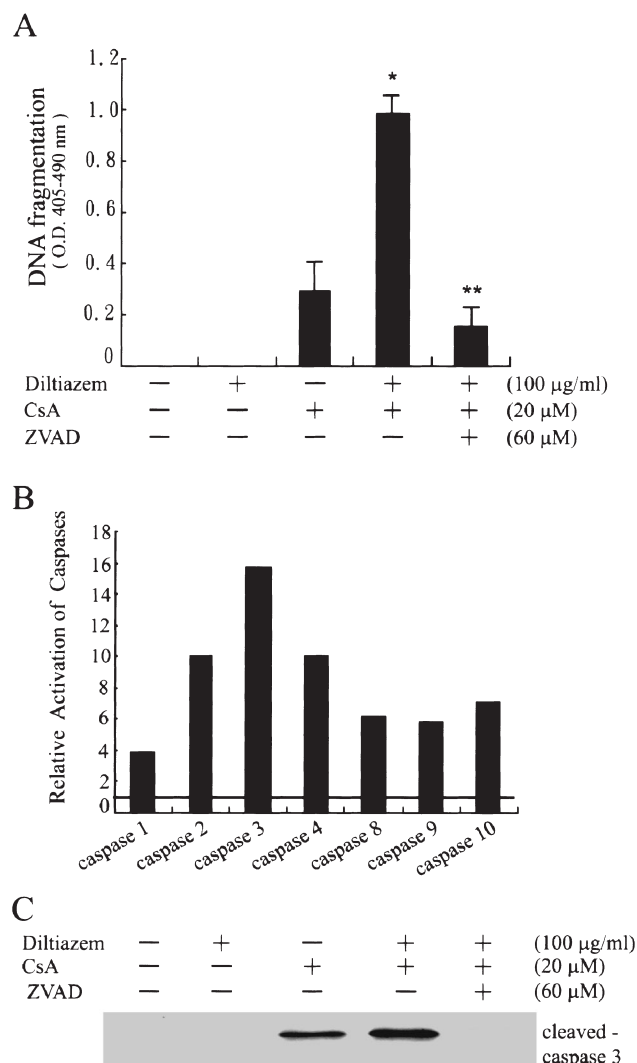


Fig. 2. Diltiazem enhancement of CsA-induced cell death results from increased apoptosis. **A**: MDCK cells were pretreated with (+) or without (–) ZVAD-fmk (60 µM) for 1 h before additions of CsA (20 µM) and/or diltiazem (100 µg/ml). 24 h later, the extent of histone-associated DNA fragments was measured using a colorimetric enzyme-immunoassay kit from Promega. The absorbance was measured at 405 nm against substrate solution as a blank at 490 nm. Experiments were performed in duplicate. Data (means ± S.E.M.) are from three independent experiments. \* $P < 0.001$  (statistically significant compared with CsA alone). \*\* $P < 0.001$  (statistically significant compared with combined treatment of CsA and diltiazem). **B**: MDCK cells were treated with or without the treatment of CsA (20 µM) and diltiazem (100 µg/ml). 24 h later, the activation of caspases was measured by colorimetric protease assay kits from BioVision. Relative activation of caspases is expressed as -fold increase compared to the level of caspase activity in control cells without treatments of CsA and diltiazem. Data are the average from two independent experiments. **C**: MDCK cells were treated as described in **A** and lysed. Equal amount of lysates was analyzed by immunoblotting with a polyclonal antibody recognizing cleaved caspase-3. The result shown is the representative of three independent experiments.

ric method (Fig. 2A). In accordance with the results in Fig. 1A, the extent of DNA fragmentation in cells treated with CsA and diltiazem was much more evident than that in cells treated with CsA alone. In addition, ZVAD-fmk effectively prevented the occurrence of DNA fragmentation induced by CsA and diltiazem. These results together indicate that en-

hancement of CsA-induced cell death by diltiazem is mainly through apoptosis. Next, the activation of caspases by CsA and diltiazem was measured using a colorimetric method, which determines the fold of increase in caspase activity compared to untreated cells (Fig. 2B). The caspases examined in our experiments all exhibited an increase in their activities. Of these, caspase-3 had the highest increase in its activity, consistent with its role as an executioner in apoptosis [19]. Because the cleavage of caspase-3 by other upstream caspases is required for its activation, the active form of caspase-3 was analyzed by immunoblotting with an antibody against the cleaved caspase-3 (Fig. 2C). More active caspase-3 was detected in cells treated with both CsA and diltiazem than in cells treated with CsA alone, suggesting a role of caspase-3 in apoptosis induced by CsA and diltiazem.

### 3.3. Overexpression of anti-apoptotic protein Bcl-2 suppresses cell death induced by CsA and diltiazem

The function of Bcl-2 is important to suppress mitochondria-dependent apoptosis [20–22]. To examine whether Bcl-2 is capable of suppressing apoptosis induced by both CsA and diltiazem, stable MDCK cell lines overexpressing Bcl-2 were established. As shown in Fig. 3A, the expression level of ectopic Bcl-2 is at least 10-fold higher than that of endogenous Bcl-2. In addition, on treatment of CsA and diltiazem, a de-

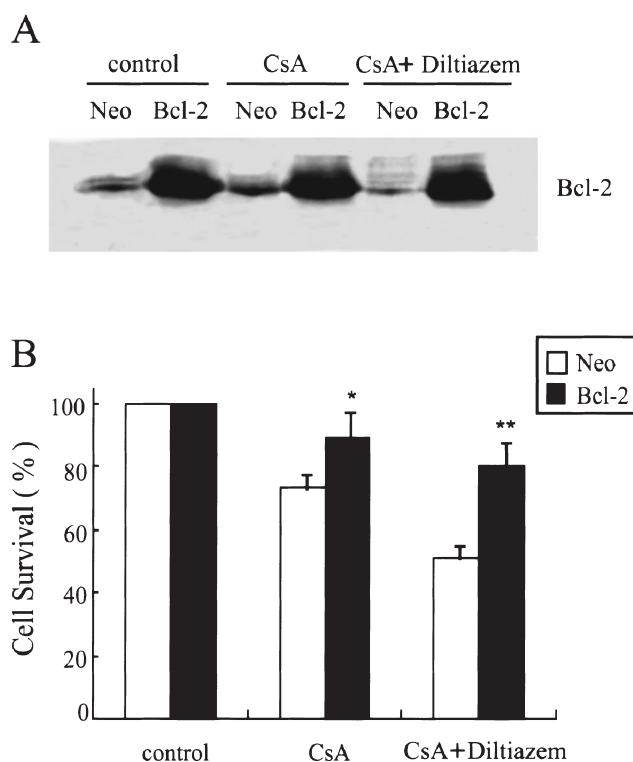


Fig. 3. Overexpression of Bcl-2 suppresses cell death induced by CsA and diltiazem. **A**: MDCK cells stably overexpressing Bcl-2 and a control clone (Neo) were treated with CsA alone or in combination with diltiazem for 24 h. Cell lysates were prepared and analyzed by immunoblotting with anti-Bcl-2. The result shown is the representative of three independent experiments. **B**: MDCK cells were treated as described in **A** and their survival was determined by trypan blue exclusion. Data (means ± S.E.M.) are from three independent experiments. \* $P < 0.05$  (statistically significant compared with CsA-treated Neo cells). \*\* $P < 0.001$  (statistically significant compared with Neo cells treated with CsA and diltiazem).

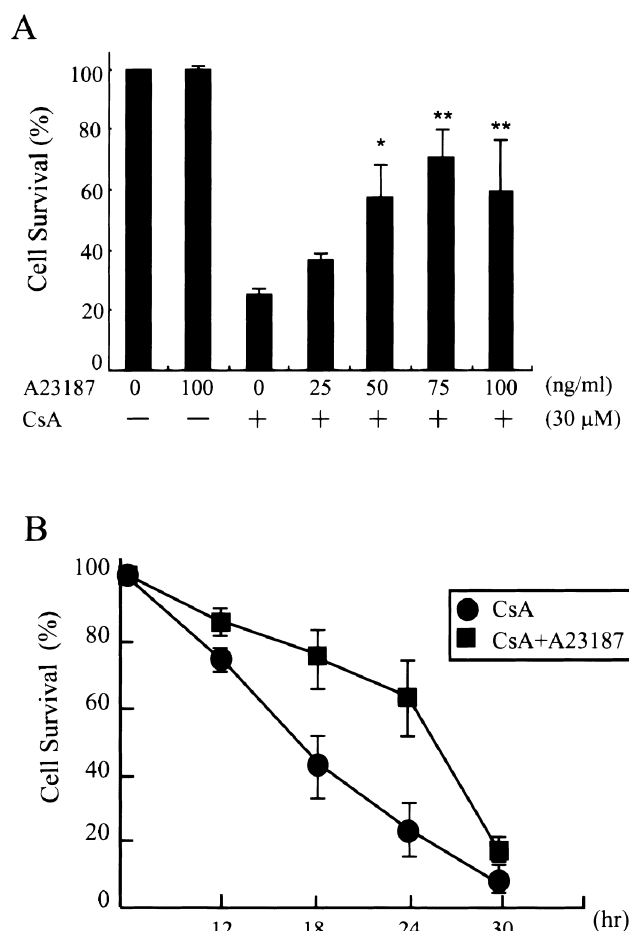


Fig. 4. Attenuation of CsA cytotoxicity by calcium ionophore A23187. A: MDCK cells which had been pretreated with various concentrations of A23187 for 1 h were treated with (+) or without (-) 30  $\mu$ M CsA. 24 h later, cell survival was determined by trypan blue exclusion. Data (means  $\pm$  S.E.M.) are from three independent experiments. When compared with CsA alone, the differences at 50, 75 and 100 ng/ml of A23187 are statistically significant. \* $P < 0.05$ . \*\* $P < 0.001$ . B: MDCK cells were treated with CsA (30  $\mu$ M) alone or in combination with A23187 (75 ng/ml). After various times as indicated, cell survival was determined by trypan blue exclusion. Data (means  $\pm$  S.E.M.) are from three independent experiments.

creased level of endogenous Bcl-2 was observed in control neomycin-resistant cells (Neo), suggesting that inhibition of Bcl-2 expression and/or cleavage of Bcl-2 protein may contribute to apoptosis induced by CsA and diltiazem. In addition, the survival rate of MDCK cells overexpressing Bcl-2 was approximately 30% higher than that of control cells in the presence of CsA and diltiazem (Fig. 3B). Together with the results in Fig. 2, it is likely that diltiazem enhancement of CsA-induced apoptosis may be mitochondria- and caspase-dependent.

#### 3.4. Attenuation of CsA cytotoxicity by calcium ionophore A23187

Because diltiazem is a calcium channel antagonist, which presumably prevents the influx of calcium from the outside of the cell and at least transiently decreases intracellular calcium level, we were interested to know whether the cytotoxicity of CsA is attenuated when the intracellular calcium is increased. To examine this possibility, the calcium ionophore

A23187, which increases the intracellular calcium, was applied to the experiments (Fig. 4). The cell survival rate of MDCK cells remained only approximately 30% after 24-h treatment with 30  $\mu$ M CsA. To our surprise, the cell survival was elevated by A23187 and most prominent when its concentration was 75 ng/ml. Moreover, in time-course experiments, 18 and 24 h after CsA treatment, the survival rate of cells with A23187 was significantly ( $\sim 40\%$ ) higher than that of cells without A23187.

#### 3.5. Suppression of CsA-induced DNA fragmentation and caspase-3 activation by calcium ionophore A23187, correlated with activation of the AKT pathway

To further confirm the effect of A23187 on attenuation of CsA cytotoxicity, the extent of DNA fragmentation and caspase-3 activation was measured. As shown in Fig. 5A, A23187 had a protective effect against CsA-induced DNA fragmentation, concomitant with a decrease in caspase-3 activation in a dose-dependent manner. The AKT pathway has been known to be a major pathway for cell survival [23–25]. To investigate whether the activation of AKT is involved in A23187 attenuation of CsA-induced apoptosis, lysates from cells treated with CsA alone or in combination with various doses of A23187 were analyzed for Ser473 phosphorylation of AKT, which serves as an indicator for AKT activation (Fig. 5B). Correlated with its ability to induce apoptosis, the treatment of CsA markedly resulted in dephosphorylation of AKT. More importantly, this dephosphorylation of AKT by CsA was prevented by A23187 in a dose-dependent manner, which was concomitant with phosphorylation of a pro-apoptotic protein, BAD. Taken together, these results suggest that calcium ionophore A23187 may suppress CsA-induced apoptosis through the AKT pathway.

#### 4. Discussion

In this study, we have used renal tubular MDCK cells as a model to examine the effect of two calcium metabolism modulators, diltiazem, a calcium channel antagonist, and A23187, a calcium ionophore, on the cytotoxicity of CsA. As in other tubular epithelial cells [10,11], CsA induced apoptosis in MDCK cells, characterized by DNA fragmentation and activation of caspases (Fig. 2). More importantly, we showed that the CsA-induced apoptosis was enhanced by diltiazem and, in contrast, attenuated by A23187, suggesting a possible regulation of CsA cytotoxicity by intracellular calcium level. In addition, the results that the tetrapeptide caspase inhibitor ZVAD-fmk (Fig. 2) or the overexpression of the anti-apoptotic protein Bcl-2 (Fig. 3) was capable of inhibiting apoptosis induced by the combined treatment of CsA and diltiazem support the involvement of caspases and mitochondria in diltiazem enhancement of CsA-induced apoptosis.

The pathogenesis of CsA nephrotoxicity has been intensively studied, which associates with endothelial injury, activation of the intrarenal rennin angiotensin system, and deposition of extracellular matrix [26]. The endothelin-mediated renal vasoconstriction has been suggested to play an important role in endothelial cell injury and the toxicity caused by CsA [27]. Therefore, it has been proposed that the favorable impact of diltiazem on CsA-treated kidney transplant recipients may be because of its effect on improving the glomerular hemodynamics and its protective effect on endothelium [13–



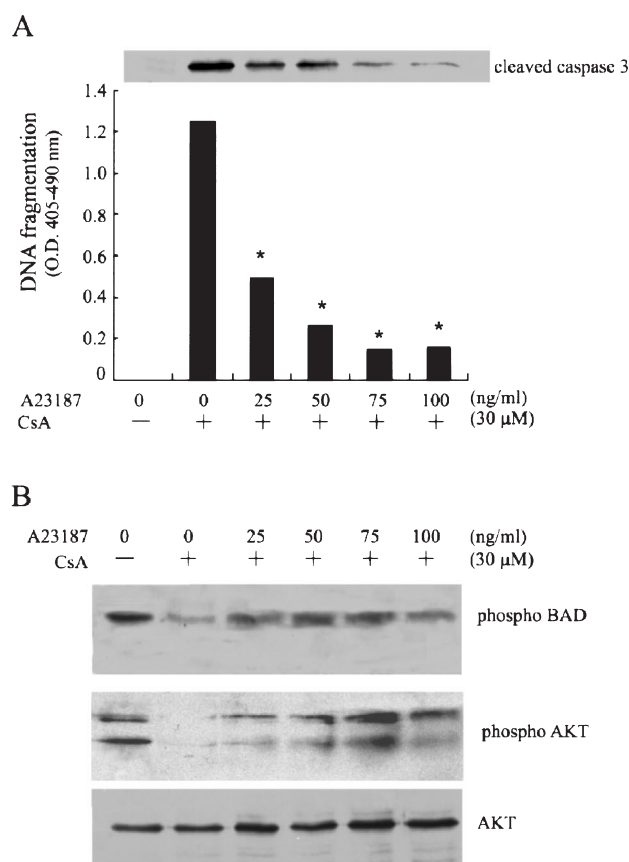


Fig. 5. Suppression of CsA-induced DNA fragmentation and caspase-3 activation by calcium ionophore A23187, correlated with activation of the AKT pathway. A: MDCK cells which had been pretreated with various doses of A23187 for 1 h were treated with (+) or without (-) 30  $\mu$ M CsA. 24 h later, the extent of histone-associated DNA fragments was measured using a colorimetric enzyme immunoassay kit from Promega. The absorbance was measured at 405 nm against substrate solution as a blank at 490 nm. Experiments were performed in duplicate. Data (means  $\pm$  S.E.M.) are the average of two independent experiments. When compared with CsA alone, the differences at 25, 50, 75 and 100 ng/ml of A23187 are statistically significant. \* $P$  < 0.001. To detect the cleaved caspase-3, equal amount of cell lysates was analyzed by immunoblotting with polyclonal anti-cleaved caspase-3. B: Cell lysates were prepared from the cells as described in A and analyzed by immunoblotting with anti-phosphoBAD, anti-phosphoAKT, and anti-AKT. The results shown are the representative of three independent experiments.

16]. Nevertheless, in this study we clearly demonstrated *in vitro* that diltiazem enhances the toxicity of CsA to renal tubular cells without consideration of endothelial effect. In fact, our results are consistent with a previous report [28] that verapamil, a calcium channel antagonist, potentiates rather than reduces the CsA toxicity. Thus, it is unlikely that the advantage of diltiazem in CsA-treated patients is through its direct 'cytoprotective' effect on renal tubular cells. Based on the assumptions described above, we propose that diltiazem may enhance the apoptotic toxicity to renal tubular cells caused by CsA in patients with endothelial dysfunction. In addition to hemodynamic mechanism, diltiazem may protect renal tissue by other mechanisms. For example, it may influence hepatic CsA metabolism and promote an increased accumulation of CsA metabolites such as M17 [29–31]. The increased level of the metabolite M17 was found to correlate with a decreased number of rejection episode [32], however,

the nephrotoxic properties and the immunological activity of the metabolite M17 remain to be defined.

Alternatively, diltiazem may exert its advantage in CsA-treated patients by enhancing immunosuppression. This possibility is based on our assumption that diltiazem may enhance the toxicity of CsA not only to renal tubular cells but also to lymphocytes. From this point of view, the synergistic effect of diltiazem and CsA on causing apoptosis in lymphocytes could enhance immunosuppression, thereby decreasing the incidence of acute and chronic rejection of kidney allografts. On the other hand, it could directly cause more severe damage in renal tubular cells, leading to chronic transplant nephropathy. Because available clinical data to these issues are few, it remains uncertain whether administration of diltiazem, in the long run, is advantageous to CsA-treated kidney transplant recipients. More studies will be necessary to elucidate the long-term effect of calcium channel antagonists on renal transplantation.

Although the mechanism of CsA-induced apoptosis in renal tubular cells is unclear, it appears to involve activation of caspases and altered expression of proteins critical for the control of apoptosis, such as p53, the Bcl-2 family proteins, and Fas/Fas-L proteins [9,11]. In this study, we show the first time that the treatment of CsA led to inactivation of the Ser/Thr protein kinase AKT, accompanied by decreased phosphorylation of its downstream target BAD (Fig. 5B). The pro-apoptotic protein BAD has been shown to dimerize with Bcl-2 and Bcl-X<sub>L</sub>, thereby inhibiting the ability of Bcl-2 and Bcl-X<sub>L</sub> to block the release of cytochrome *c* from mitochondria, a critical step in the activation of the downstream caspase protease cascade [21,33,34]. Thus, although the mechanism for CsA to inactivate AKT is currently unknown, our finding provides a possible explanation for CsA to activate caspases, as described in this report and elsewhere [9,10]. Moreover, we found that, correlated with its ability to rescue cells from CsA-induced apoptosis, the calcium ionophore A23187 prevented the CsA-caused inactivation of AKT through an unknown mechanism, concomitant with increased phosphorylation of BAD (Fig. 5). This finding accords with the notion that phosphorylated BAD by activated AKT is translocated from the mitochondria to the cytosol through its association with 14-3-3, allowing Bcl-X<sub>L</sub> to exert its function as a gatekeeper of cytochrome *c* efflux at mitochondria membrane and to promote cell survival [35,36]. Recently, Fornoni et al. [37] reported that CsA induces a decreased level of Bcl-X<sub>L</sub> and apoptosis in a murine podocyte cell line, which can be prevented by hepatocyte growth factor through the AKT pathway.

In T lymphocytes, calcium initiates the proliferative response by stimulating various transcription factors such as NF- $\kappa$ B, NFAT, and CREB. One action of calcium is to stimulate the calcium-dependent protein phosphatase calcineurin to dephosphorylate NFAT, which then enters the nucleus [38]. CsA blocks the calcineurin-dependent dephosphorylation of NFAT through its inhibitory effect on calcineurin [39,40]. It is not known whether in renal tubular cells, the calcineurin/NFAT pathway is a target of CsA and whether its inactivation by CsA contributes to CsA-induced apoptosis. Recently, Huang et al. [41] showed that vanadium activates NFAT in mouse fibroblasts or epidermal cells, which can be inhibited by a calcium channel antagonist, nifedipine, and enhanced by two calcium ionophores, A23187 and ionomycin. Their results

suggest a possible role of intracellular calcium in NFAT activation in non-lymphocytes. Experiments are in progress to examine the effects of CsA and calcium metabolism modulators on NFAT activation and its link with CsA-induced apoptosis in renal tubular cells.

In summary, this *in vitro* study indicates that diltiazem enhances the apoptosis of renal tubular cells caused by CsA and suggests that the inhibition of the AKT pathway may be critical for CsA to induce apoptosis.

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