Protective effect of 2-deoxy-D-glucose on the cytotoxicity of cyclosporin A *in vitro*

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Abstract. The present study aimed to investigate the mechanism underlying the protective effect of 2-deoxy-D-glucose (2-DG) on the cytotoxicity of cyclosporin A (CsA) *in vitro* using NRK-52E cells. Staining with Hoechst 33342/propidium iodide prior to flow cytometric analysis was performed to assess the rate of cellular apoptosis and necrosis induced by CsA. The expression levels of lactate dehydrogenase (LDH), caspase 3, receptor-interacting protein kinase 3 (RIP3), reactive oxygen species (ROS), glutathione (GSH) and malondialdehyde (MDA) were detected using colorimetry, ELISA, western blotting or flow cytometric analysis to determine the protective effects of 2-DG on CsA-induced cell death. The results demonstrated that 2-DG inhibited the release of LDH, the activation of caspase 3 and the generation of ROS induced by CsA, but had no effect on the expression of RIP3. Treatment with 2-DG increased the expression of GSH and decreased the expression of MDA in dose-dependent manner, and reduced the rate of the cellular apoptosis and necrosis induced by CsA. Therefore, 2-DG inhibited CsA-induced cellular apoptosis and necrosis, possibly by reducing the production of ROS. Inhibiting the activation of caspase 3 is one of the protective mechanisms of 2-DG, however, the expression of RIP3 remained unaltered following treatment with 2-DG. Whether 2-DG inhibits the CsA-induced necrosis and apoptosis by inhibiting the RIP3 signaling pathway remains to be elucidated.

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Introduction

Cyclosporin A (CsA), which was initially isolated from the fungus *Tolypocladium inflatum* (*Beauveria nivea*) by Hans Peter Frey in a soil sample obtained in 1969 from Hardangervidda, Norway (1), is an effective immunosuppressant, which is widely used against graft rejection and has clinical applications in the treatment of various autoimmune disorders (2-5). However, its application has been limited due to the severe toxicity caused by CsA, particularly in patients with renal injury (6,7). At present, in order to reduce the CsA-induced toxicity, the plasma concentration of CsA is adjusted and maintained at a non-toxic range by monitoring the drug concentration in the patient's blood, however this approach required specialized equipment and professional staff (7). Therefore, investigations have attempted to identify effective components from other drugs or natural sources, which inhibit the renal toxicity of CsA. Protection against nephrotoxicity induced by CsA was observed in several reagents, including shallot, black grape, garlic extracts (8,9), L-arginine, N-acetylcysteine, vitamin E and curcumin (10-13). Certain reagents are involved in the inhibition of glycolysis, therefore, the present study investigated whether 2-deoxy-D-glucose (2-DG), a typical inhibitor of glycolysis, can protect against CsA-induced nephrotoxicity.

2-DG is a type of deoxyglucose and exists in all types of microbes. Its predominant role is to inhibit glycolysis, which induces cell death in tumor cells and has a protective effect on normal cells. Therefore, 2-DG has been widely investigated as an adjuvant of antitumor drugs (14,15). The protective mechanism of 2-DG may be associated with it increasing the expression levels of the heat shock protein (HSP)70 stress protein and phosphorylated-AKT, and inhibiting the production of reactive oxygen species (ROS) (16-18). The present study investigated the protective mechanism of 2-DG on the cytotoxicity of CsA *in vitro*.

Materials and methods

Cell culture. The rat tubular cell line, NRK-52E, was cultured in 6-, 24- or 96-well plates with America Type Culture Collection-modified Dulbecco's modified Eagle's medium

(DMEM), containing 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, which was supplemented with 10% fetal bovine serum (Takara Biotech Co., Ltd., Dalian, China), 100 U/ml penicillin and 100 U/ml streptomycin (TaKaRa Biotech Co., Ltd.) at 37˚C with 5% CO_2.

Determination of lactate dehydrogenase (LDH) activity. LDH activity was determined using an LDH assay kit (Beyotime Institue of Biotechnology Co. Ltd., Shanghai, China), according to the manufacturer's instructions. Briefly, the NRK-52E cells were seeded into 24-well plates at a density of $2x10^5$ cells/well in DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was subsequently replaced with DMEM without 10% fetal bovine serum, and the cells were cultured for 12 h prior to replacing the medium again and pretreating the cells with 2-DG (2, 10 and 25 mM), Nec-1 (50 μM) and z-VAD-fmk (20 μM; Beyotime Institute of Biotechnology, Co. Ltd.) for 30 min. CsA (10 μM; Beyotime Institute of Biotechnology, Co. Ltd.) was added to the cells and cultured at 37˚C for 24 h. The cell culture supernatants were collected by centrifugation at 15,000 x g for 5 min and the LDH levels were measured spectrophotometrically by nicotinamide adenine dinucleotide oxidation at 440 nm on a PhotoLab 6100.

Hoechst 33342/propidium iodide (PI) double staining. The rates of cellular apoptosis or necrosis were assessed with Hoechst 33342/PI staining. Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was replaced with DMEM without 10% fetal bovine serum, and the cells were cultured for 12 h prior to replacing the medium and pretreating with 2-DG (25 mM), Nec-1 (50 μM) and z-VAD-fmk (20 μM) for 30 min. CsA (10 μM) was added and the cells were cultured at 37˚C for 24 h. The cells were subsequently stained with Hoechst 33342 and PI (10 μM) for 15 min. The stained cells were observed under an IX71 inverted fluorescence microscope (Olympus IX710; Olympus, Tokyo, Japan).

Analysis of apoptosis and necrosis by flow cytometry. Apoptotic and necrotic cell death were assessed using an annexin V-fluorescein isothiocyanate (FITC)-conjugated)/PI apoptosis kit and a fluorescent activated cell sorter (FACS) flow cytometer (BD, San Jose, CA, USA). Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was replaced with DMEM without serum and the cells were cultured at 37˚C for a further 12 h. The medium was replaced again, and the cells were pretreated with 2-DG (25 mM), Nec-1 (50 μM) and z-VAD-fmk (20 μM) for 30 min prior to the addition of 10 μM CsA and the cells were cultured for 24 h. The cells were harvested and resuspended in 500 μl binding buffer. The cells were incubated with 5 μl annexin V-FITC in the dark at 37˚C for 15 min prior to the addition of 10 μl PI (50 mg/ml) to the cell suspension and incubation for an additional 5 min. The cells were immediately analyzed using a FACSCalibur flow cytometer. For all samples, the fluorescence of 10,000 cells was gated and quantified. The percentages of the cells in the lower right (early apoptotic cells) and upper right (late apoptotic/necrotic cells) region of the scatter plot of annexin V-FITC were calculated using CellQuest software (BD Biosciences) for comparison.

Western blotting. The expression levels of caspase 3 and receptor-interacting protein kinase 3 (RIP3) were assessed by western blotting. Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was replaced with DMEM without serum and cultured at 37˚C for 12 h. The medium was replaced again, and the cells were pretreated with 2-DG (25 mM), Nec-1 (50 μM) and z-VAD-fmk (20 μM) for 30 min prior to the addition of 10 μM CsA and the cells were cultured at 37˚C for 24 h. The cells were washed twice with cold phosphate-buffered saline (PBS; Takara Biotech Co., Ltd.) and lysed with radioimmunoprecipitation buffer, containing 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μg/ml leupeptin, 1.5 mM EDTA, 1 mM NaVanadate, for 25 min on ice. The lysates were centrifuged at 15,000 x g for 10 min at 4˚C and the protein concentrations were detected using a Bradford protein assay (Beyotime Institute of Biotechnology Co. Ltd.). Briefly, a series of protein standards were diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 250, 500, 750 and 1,500 μg/ml. Serial dilutions of the unknown sample to be measured were also prepared. The standards and samples (100 μl) were added to a separate test tube and 5 ml Coomassie Blue was added to each tube, and vortexed. The samples were measured at 595 nm wavelength and the absorbance of the standards, vs. their concentration was plotted. The extinction coefficient was calculated and the concentrations of the unknown samples were determined. The total proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Takara Biotech Co., Ltd.) and were transferred onto polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20 (TBST; Takara Biotech Co., Ltd.) for 2 h and were then incubated with the following primary antibodies: Rabbit anti-RIP3 (1:1,000; Antibodies-online Inc., Atlanta, GA, USA, cat. no. ABIN360159), anti-cleaved caspase 3 rabbit polyclonal (1:1,000; Trevigen, Inc, Gaithersburg, MD, USA, cat. no. 2305-PC-020) or mouse anti-tubulin (1:10,000; Antibodies-online Inc; cat. no. ABIN125953) at 4˚C overnight. The membranes were incubated with secondary antibodies conjugated to horseradish peroxidase at room temperature for 1 h. Antibody binding was detected using an enhanced chemiluminescence detection kit (Beyotime Institute of Biotechnology Co. Ltd.). The images were analyzed using Quantity One software (Bio-Rad).

Determination of ROS. The effect of 2-DG on the production of ROS induced by CsA was evaluated using flow cytometry. Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was replaced with DMEM without serum and the cells were cultured at 37˚C for a further 12 h. The medium was changed and the cells were treated with 2-DG (25 mM) for 30 min prior to the addition of 10 μM CsA. Rosup was added 30 min prior to cell digestion. The cells were incubated at 37˚C for 12 h, and changes in the levels of ROS were detected using flow cytometry.

Determination of glutathione (GSH). GSH was detected using a GSH assay kit (Beyotime Institute of Biotechnology Co. Ltd.),

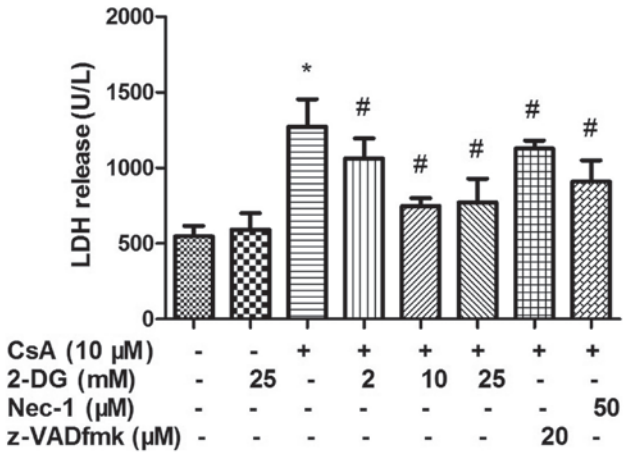

Figure 1. Effect of 2-DG on the inhibition of LDH release induced by CsA. The data are expressed as the mean ± standard deviation (*P<0.05, vs. untreated control group; $^{\#}$P<0.05, vs. CsA group). CsA, cyclosporin A; LDH, lactate dehydrogenase, 2-DG, 2-deoxy-D-glucose.

according to the manufacturer's instructions. Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum, and cultured at 37˚C for 24 h. The medium was replaced with DMEM without serum and the cells were cultured at 37˚C for a further 12 h. The medium was replaced again, and the cells were treated with 2-DG (25 mM) for 30 min prior to the addition of 10 μM CsA and culture for 24 h. The cells were then washed with cold PBS and lysed. The supernatant was assessed using an ELISA reader to determine the absorbance at the wavelength of 405 nm.

Determination of malondialdehyde (MDA). MDA is a natural product of lipid oxidation, therefore, the levels of MDA represent the lipid peroxidation level. MDA was detected using a lipid peroxidation MDA assay kit (Beyotime Institute of Biotechnology Co. Ltd.). Briefly, $5x10^5$ cells/well were seeded into 6-well plates with DMEM, containing 10% fetal bovine serum and cultured at 37˚C for 24 h. The medium was replaced with DMEM without serum and the cells were cultured at 37˚C for 12 h. The medium was replaced again and the cells were treated with 2-DG (25 mM) for 30 min, prior to the addition of 10 μM CsA and cultured at 37˚C for 24 h. The cells were subsequently washed with cold PBS and lysed with radioimmunoprecipitation buffer. The supernatant was assessed using an ELISA reader (Peiou 318C) to determine the absorbance at a wavelength of 532nm and 600nm.

Data analysis. All data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The differences between experimental groups were analyzed by one-way analysis of variance and Kruskal-Wallis tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of 2-DG on LDH. As shown in Fig. 1, treatment with 2, 10 or 25 mM 2-DG significantly inhibited the release of LDH induced by CsA. The protective effects of 10 and 25 mM 2-DG were higher compared with that of 50 μM Nec-1

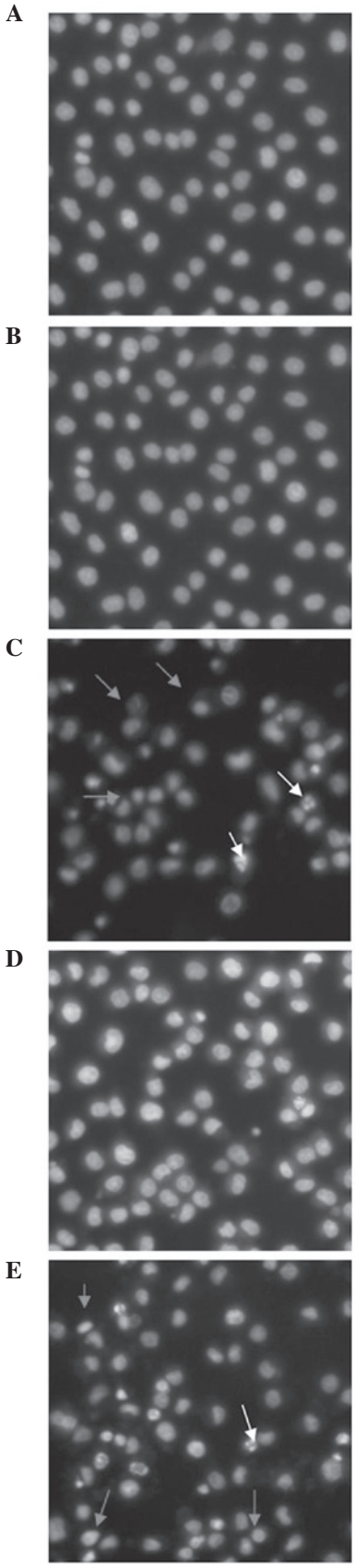

Figure 2. Effect of 2-DG on CsA-induced necrosis, detected by Hoechst 33342/propidium iodide double staining (magnification, x200). (A) Untreated cells (control); (B) cells treated with 25 mM 2-DG for 24 h; (C) cells were treated with 10 μM CsA for 24 h; (D) pretreatment with 25 mM 2-DG 30 min prior to CsA treatment; (E) pretreatment with 20 μM z-VAD-fmk 30 min prior to CsA treatment. White arrow, apoptosis; grey arrow, necrosis. CsA, cyclosporin A; LDH, lactate dehydrogenase; 2-DG, 2-deoxy-D-glucose.

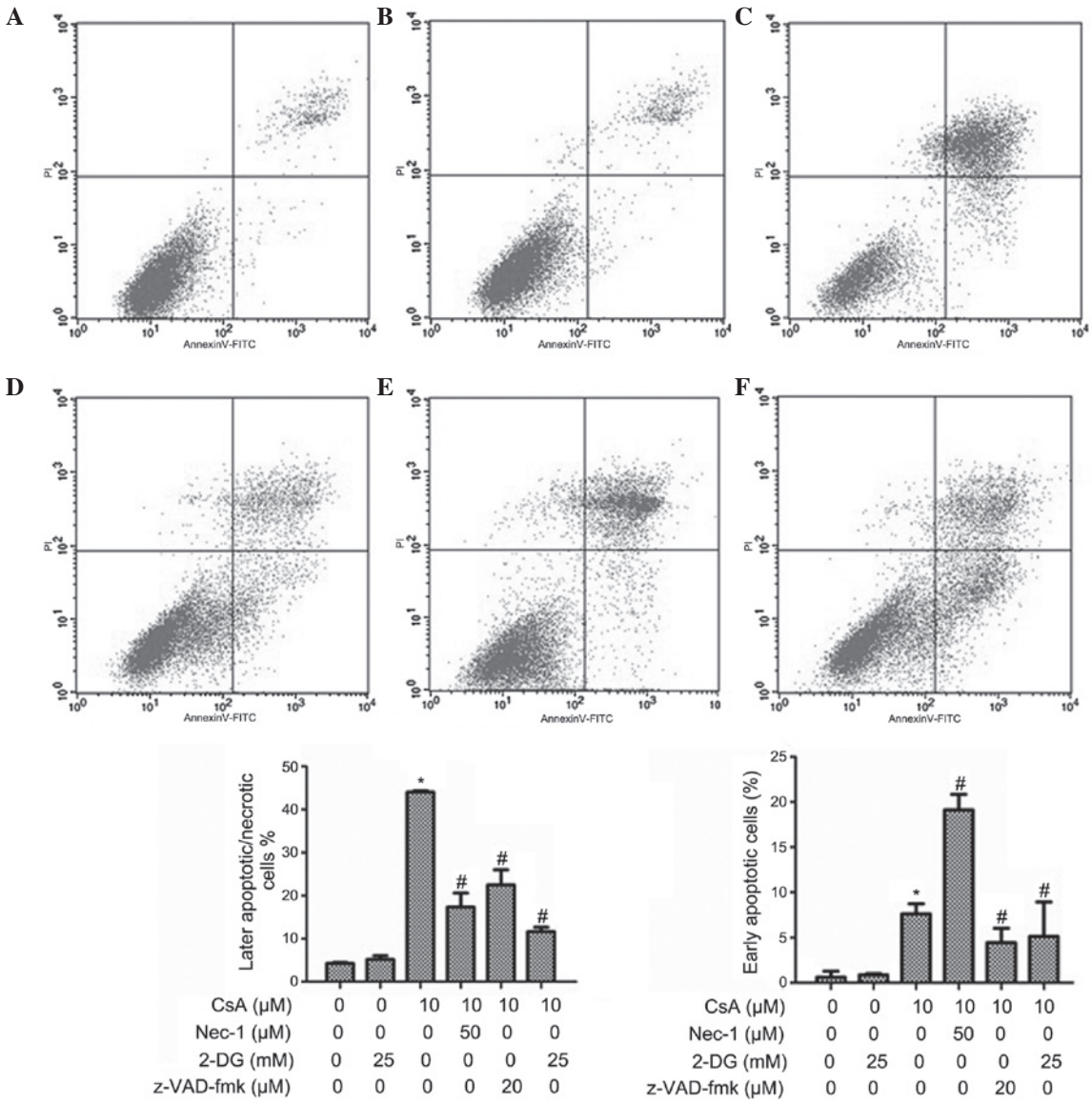

Figure 3. Effect of 2-DG on CsA-induced necrosis and apoptosis. The rates of apoptosis and necrosis were analyzed by flow cytometric analysis. The experimental groups were divided into (A) untreated cells (control), (B) cells treated with 25 mM 2-DG for 24 h, (C) cells were treated with 10 μM CsA for 24 h, (D) pretreatment with 25 mM 2-DG 30 min prior to treatment with CsA, (E) pretreatment with 50 μM Nec-1 30 min prior to treatment with CsA and (F) pretreatment with 20 μM z-VAD-fmk 30 min prior to treatment with CsA. In the graphs of the results, data are expressed as the mean ± standard deviation (*P<0.05, vs. control group; $^{\#}$P<0.05, vs. CsA group). CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose; FITC, fluorescein isothiocyanate; PI, propidium iodide.

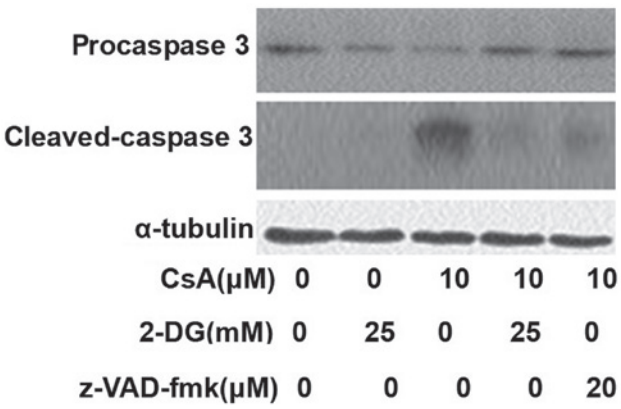

Figure 4. Western blotting results demonstrating inhibitory effect of 2-DG on caspase 3. Western blotting was performed to investigate the effect of CsA on the cleavage of procaspase 3 into activated caspase 3 and its inhibition by 2-DG. α-tubulin was used as an internal loading control. CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose.

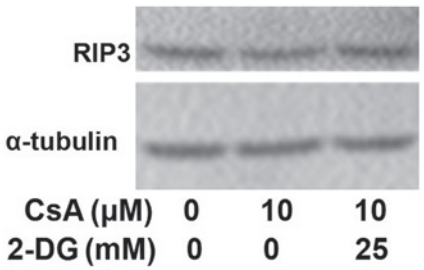

Figure 5. Western blotting results demonstrating the inhibitory effect of 2-DG on RIP3. The expression of RIP3 was detected by western blotting. α-tubulin was used as an internal loading control. CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose; RIP, receptor-interacting protein kinase.

and 20 μM z-VAD-fmk. However, no difference was observed between 10 and 25 mM 2-DG.

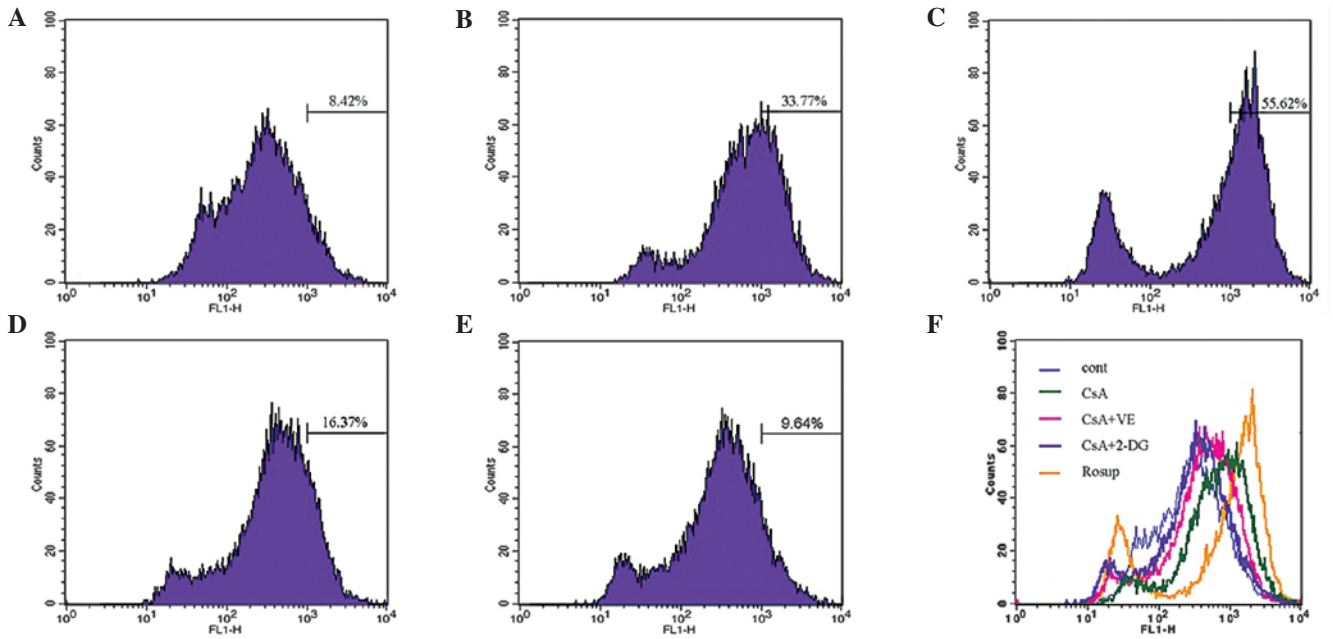

Figure 6. Effect of 2-DG on CsA-induced production of ROS in NRK-52E cells. The effect of 2-DG on the production of ROS induced by CsA was evaluated by flow cytometry. (A) Untreated control; (B) CsA (10 μM) treatment; (C) Rosup treatment; (D) VE (100 μM) and CsA (10 μM) treatment; (E) 2-DG (25 mM) and CsA (10 μM) treatmen; (F) CsA of blue line demonstrated in a significant increase in the generation of ROS and 2-DG suppressed the generation of ROS. The data were expressed as the percentage of cells positive for the ROS sensitive fluorometric probe, 2, 7-dichlorofluorescein. CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose; ROS, reactive oxygen species.

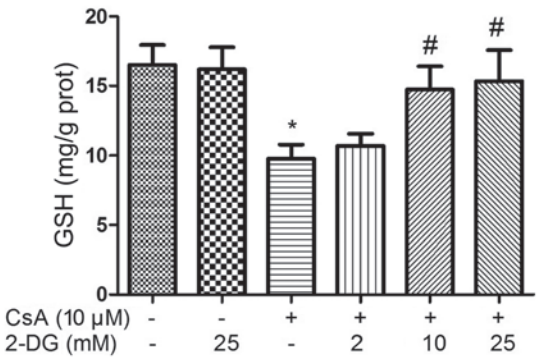

Figure 7. Effect of 2-DG on the cellular CsA-induced expression of GSH in NRK-52E cells. The intracellular expression of GSH was determined by monochloribimane derivation in NRK-52E cells, either untreated (control) or treated with 10 μM CsA alone, 2-DG alone or the two in combination for 24 h. Data are expressed as the mean ± standard deviation (*P<0.05, vs. control; #P<0.05, vs. CsA). CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose; GSH, glutathione.

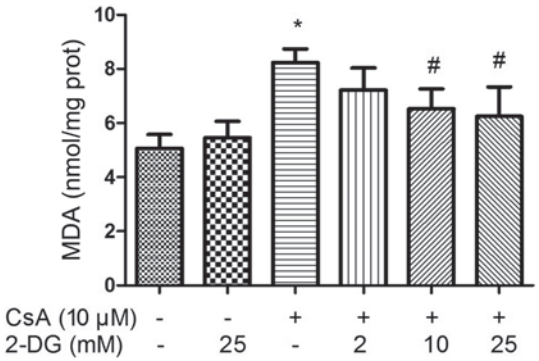

Figure 8. Effect of 2-DG on the cellular CsA-induced expression of MDA in NRK-52E cells. The intracellular expression of MDA was determined by thiobarbituric acid reagent in NRK-52E cells, either untreated (control) or treated with 10 μM CsA alone, 2-DG alone or the two in combination for 24 h. The data are expressed as the mean ± standard deviation (*P<0.05, vs. control; #P<0.05, vs. CsA group). CsA, cyclosporin A; 2-DG, 2-deoxy-D-glucose; MDA, malondialdehyde.

Hoechst 33342/PI double staining. The morphological changes of apoptotic and necrotic cells were observed by Hoechst 33342/PI double staining. As shown in Fig. 2, treatment with 25 mM 2-DG was not toxic to the NRK-52E cells. This treatment reduced the rate of CsA-induced cellular apoptosis and necrosis, and the inhibition of apoptosis was similar to that of z-VAD-fmk.

Flow cytometric analysis of the effects of 2-DG on necrosis and apotosis. The results of the flow cytometry were the same as those obtained by Hoechst33342/PI double staining. Treatment with 25 mM 2-DG reduced the rate of CsA-induced cellular apoptosis and necrosis. The inhibitory effect of 25 mM 2-DG on cellular apoptosis and necrosis was higher compared with treatment with 50 μM Nec-1 and was similar to that of z-VAD-fmk (Fig. 3).

Effects of 2-DG on the activation of caspase 3. Caspase 3 was activated by CsA and this activation was inhibited by treatment with 2-DG. Treatment with z-VAD-fmk also inhibited the activation of caspase 3 (Fig. 4).

Effects of 2-DG on the expression of RIP3. Western blot analysis was performed to investigate the effect of 2-DG on the expression of RIP3. The results demonstrated that 2-DG had no effect on the expression of RIP3 (Fig. 5).

Effect of 2-DG on the production of CsA-induced ROS. The effect of 2-DG on the production of ROS, induced by CsA, was assessed using flow cytometry. Treatment with CsA revealed a significant increase in the generation of ROS, whereas treatment with 2-DG suppressed the generation of ROS. As a ROS scavenger, vitamin E decreased the cellular level of ROS following CsA treatment. The effects of 2-DG were more marked compared with that of VE (Fig. 6).

Effect of 2-DG on cellular levels of CsA-induced GSH. The intracellular levels of GSH decreased following treatment with CsA for 12 h. By contrast, the intracellular level of GSH increased in the cells pre-treated with 2-DG prior to CsA treatment, which occurred in a dose-dependent manner (Fig. 7).

Effect of 2-DG on the CsA-induced cellular levels of MDA. The intracellular levels of MDA increased following treatment with CsA for 12 h. By contrast, the levels of MDA in the cells decreased following pre-treatment with 2-DG prior to treatment with CsA, which occurred in a dose-dependent manner (Fig. 8).

Discussion

2-DG is an inhibitor of glucose transport and glycolysis, and is present naturally in bacteria. At present, 2-DG is predominantly used in the treatment of tumor cells, as it has a selective effect on tumor cells and is non-toxic to normal cells at the conventional dose (15,19). The present study hypothesized that this may be associated with the inhibition of glycolysis. LDH is an enzyme widely present in the cytosol. When the plasma membrane integrity is disrupted, LDH leaks into the culture media and its extracellular level is increased (20). In the present study, the protective effect of 2-DG on CsA-induced cellular toxicity was investigated. The present study revealed that 2-DG inhibited the release of LDH in the CsA-induced NRK-52E cells, which suggested that 2-DG inhibited CsA-induced cellular necrosis and apoptosis. The results of the flow cytometric and morphological analyses confirmed that 2-DG almost completely inhibited the toxicity of CsA on the NRK-52E cells, which may be indirect evidence that acceleration in the rate of cellular glycolysis by CsA is the predominant reason for its cytotoxicity.

ROS is a by-product of the oxidative respiratory chain and is produced predominantly in the mitochondria, which are rich in renal tubular epithelial cells (21). A low level of ROS can eliminate the invading bacteria, however, excess ROS can lead to cell dysfunction and cause cellular apoptosis or necrosis (21-23). The present study found that 2-DG reduced the increased production of ROS, which was induced by CsA. Reducing the production of ROS may be the predominant mechanism underlying the effect of 2-DG against CsA cytotoxicity. In addition, the levels of the intracellular antioxidant, GSH, and the oxidation product, MDA, were determined. This revealed that 2-DG inhibited the CsA-induced reduction in GSH and increase in MDA content, which demonstrated that CsA altered the cellular redox status and 2-DG enabled the cell recovery to the normal level.

The present study also revealed that CsA activated and cleaved caspase-3, whereas 2-DG inhibited this activation. This suggested that the inhibitory action of 2-DG on the activity of caspase was also involved in the protective effects on CsA-induced toxicity.

The importance of RIP3 in the signal transduction cascade of necrosis has been reported (24,25). In our previous study, RIP3 and ROS were found to be involved in CsA-induced necroptosis (21). However, 2-DG did not alter the expression of RIP3 in the present study, therefore, whether 2-DG inhibits the activity of the RIP3 phosphokinase and the necroptosis induced by CsA remains to be elucidated.

In conclusions, 2-DG inhibited CsA-induced cellular apoptosis and necroptosis, which may be through reducing the CsA-induced production of ROS. The inhibition of caspase 3 activation was demonstrated as one of the protective mechanism of 2-DG, however, the expression of RIP3 was not affected. Whether 2-DG affects the phosphorylation of RIP3 and inhibits CsA-induced necroptosis through inhibition of the RIP3 signaling pathway remains to be elucidated.

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