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Cyclosporin A inhibits H_2O_2-induced apoptosis of human fibroblasts

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Abstract Several clinical studies have shown that cyclosporin A (CsA) is effective for treating a variety of chronic inflammatory and autoimmune diseases. Because reactive oxygen species are believed to play a key role in the development of these diseases, causing cell apoptosis, we investigated whether CsA inhibits H_2O_2-induced apoptosis. Preincubation of human fibroblasts with CsA dose-dependently decreased H_2O_2-induced apoptosis. Apoptosis suppression by CsA was correlated with the prevention of mitochondrial dysfunction and caspase activation. Thus, our results suggest that the inhibition of apoptosis by CsA may at least partly contribute to the anti-inflammatory effect of CsA.

Key words: Apoptosis; Cyclosporin A; Fibroblast; Hydrogen peroxide

1. Introduction

Reactive oxygen species (ROS) have been implicated in the pathogenesis of many inflammatory diseases such as rheumatoid arthritis, acute respiratory distress syndrome, and periodontal disease [1,2]. Phagocytic cells are a major source of ROS, which are potent inducers of programmed cell death (apoptosis) at sites of inflammation [3]. One prominent effect of ROS is to activate the mitochondrial permeability transition pore and release the mitochondrial protein cytochrome *c*. In the cytosol, cytochrome *c* in combination with Apaf-1 activates caspase-9, which finally leads to activation of caspase-3 and DNA fragmentation [4–11].

Several experimental and clinical studies have suggested that cyclosporin A (CsA), which is commonly used as an immunosuppressive agent to prevent organ transplant rejection, is effective for treating a variety of chronic inflammatory and autoimmune disorders such as psoriasis, asthma, ulcerative colitis, and rheumatoid arthritis [12,13]. While some of the benefits of these treatments can be attributed to suppressive actions against T cells, neutrophils, eosinophils, monocytes and fibroblasts [14–19], it has been shown that there may be additional effects on apoptosis [20–24].

Thus, the aim of the present study was to investigate the influence of CsA on H_2O_2-induced apoptosis of human fibroblasts. Our results demonstrated that CsA reduced H_2O_2-induced cytochrome *c* release, caspase-3 activation, and subsequent DNA fragmentation. The present findings suggest that the ability of CsA to act as an anti-inflammatory drug may be due partly to its direct effects on apoptosis.

2. Materials and methods

2.1. Cell culture

Human gingival fibroblasts established from three patients with healthy gingival tissues were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin and 100 μg of streptomycin per ml in a humidified atmosphere of 5% CO_2 at 37°C. Cells used in this study were at the fifth to fifteenth passage. CsA was donated by Novartis Pharma Inc. (Basel, Switzerland), dissolved in ethanol, and added to the culture medium. The final concentration of ethanol was 0.01%.

2.2. DNA fragmentation analysis

After incubation of the cells with H_2O_2 for 24 h, cells were scraped off the plates and pelleted by centrifugation. The pellet was treated with 100 μl of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, Triton X-100) for 10 min at 4°C. After centrifugation at 1500 rpm for 20 min, the supernatant was collected and treated for 1 h with RNase A (final concentration 0.4 μg/ml) at 37°C followed by digestion with proteinase K (final concentration 0.4 μg/ml). The DNA was separated by electrophoresis on 1.5% agarose gels. The gels were then stained with ethidium bromide and visualized under ultraviolet light.

2.3. Western blot analysis

To evaluate mitochondrial cytochrome *c* release, cytosolic protein extracts were obtained as described previously [9]. Cells were washed twice with PBS and the cell pellets were resuspended in ice-cold buffer containing 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF. The lysate was centrifuged at $10\,000 \times g$ for 10 min, and the resulting supernatant was further centrifuged for 20 min. Sample proteins were separated by SDS-PAGE in 15% acrylamide gels, followed by Western blotting. The membranes were probed with antibodies against cytochrome *c* (Pharmingen, San Diego, CA, USA), followed by examination with an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA) and autoradiography.

2.4. Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

For determination of mitochondrial function, cells were incubated for 60 min at 37°C with MTT at a final concentration of 2.0 μg/ml, then lysed and the absorbance at 570 nm measured.

2.5. Caspase activity

Caspase activity was measured using a caspase-3 colorimetric protease assay kit according to the manufacturer's protocol (MBL, Nagoya, Japan).

Absorbance was converted to pmol of nitroanilide using a standard curve generated with free nitroanilide.

3. Results and discussion

3.1. Effect of CsA on H_2O_2-induced DNA fragmentation of fibroblasts

Apoptosis, in contrast to necrosis, is characterized by a specific series of intracellular events leading ultimately to DNA fragmentation. To test the effect of CsA on apoptosis, we first examined DNA fragmentation. Exposure of fibroblasts to 50 μM H_2O_2 for 24 h revealed DNA fragmentation. Pretreatment with CsA dose-dependently reduced H_2O_2-in-

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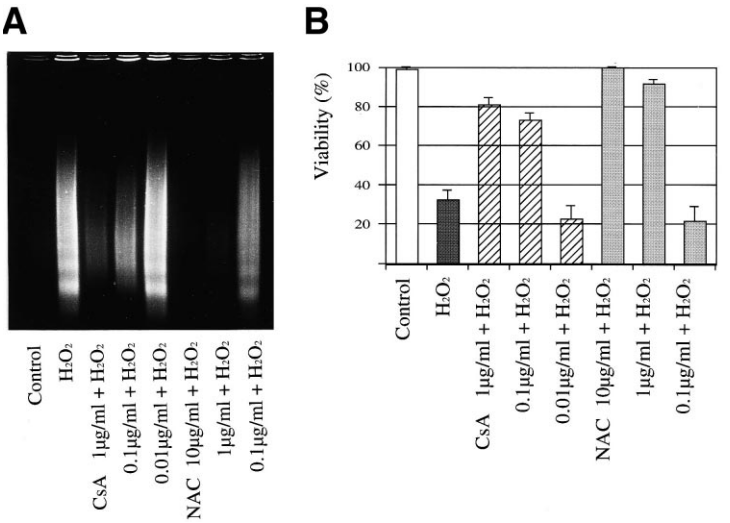

Fig. 1. A: Effect of CsA on H_2O_2-induced DNA fragmentaion in fibroblasts. Cells were preincubated with CsA for 10 min or NAC for 1 h before exposure to 50 μM H_2O_2. DNA was extracted 24 h after H_2O_2 exposure and analyzed by electrophoresis on a 1.5% agarose gel. DNA was visualized with ethidium bromide. Results are representative of three independent experiments. B: Viability was determined by trypan blue staining 24 h after H_2O_2 exposure. The results are presented as means of three independent experiments.

duced DNA fragmentation (Fig. 1). Control experiments using *N*-acetylcysteine (NAC), a well known antioxidant, also showed an anti-apoptotic effect.

3.2. Effect of CsA on mitochondrial function

To assess the physiological state of the mitochondria, MTT reduction by the cells treated with H_2O_2 was measured. MTT reduction by succinate dehydrogenase, a component of complex II of the respiratory chain, is an indicator of mitochondrial function [25]. When cells were exposed to H_2O_2 for 6 h, a decrease in MTT reduction was observed (Fig. 2A). Pretreatment with CsA dose-dependently reversed the decrease of MTT reduction. In control experiments, NAC also protected the cells from loss of MTT reduction.

Disruption of mitochondrial function results in specific release of the mitochondrial enzyme cytochrome *c* into the cytosol [26–29]. Therefore, the cytosolic fraction was prepared, and cytochrome *c* was detected by Western blotting. As shown in Fig. 2B, incubation of the cells with H_2O_2 for 6 h induced the release of the cytochrome *c* into the cytosolic fraction. Preincubation with CsA dose-dependently suppressed the release of cytochrome *c* (Fig. 2C). NAC also prevented H_2O_2-induced cytochrome *c* release.

3.3. Effect of CsA on caspase activity

Caspase activity in the H_2O_2-treated cells was measured using DEVD peptide conjugated to nitroanilide. After 18 h of exposure to H_2O_2, there was a significant increase in the rate of DEVD-*p*NA cleavage compared to the control cells (Fig. 3A). Preincubation with CsA dose-dependently inhibited caspase activity (Fig. 3B), reaching 67% inhibition at 1.0 μg/ml CsA. Pretreatment with 10 mM NAC completely inhibited caspase activity (98% inhibition), confirming that oxidative stress induced caspase activity in this system.

The present investigation has demonstrated that H_2O_2 induces dysfunction of mitochondria, leading to release of cytochrome *c* into the cytosol, and thereby stimulating apoptosis of human fibroblasts. The results also confirm that CsA inhibits H_2O_2-induced apoptosis of human fibroblasts. The mechanism underlying the protective effect of CsA appears to involve stabilization of mitochondria, as demonstrated by the prevention of decreased MTT reduction and cytochrome *c* release. It has been reported that CsA is a specific inhibitor of the mitochondrial permeability transition pore responsible for cytochrome *c* release, and thus an essential apoptosis-inducing factor [21,24,25]. This modification of mitochondria by CsA can provide an explanation for the inhibitory effect of CsA on H_2O_2-induced apoptosis of human fibroblasts. In conclusion, our results suggest that the inhibition of apoptosis by CsA may at least partly contribute to the anti-inflammatory effect of CsA.

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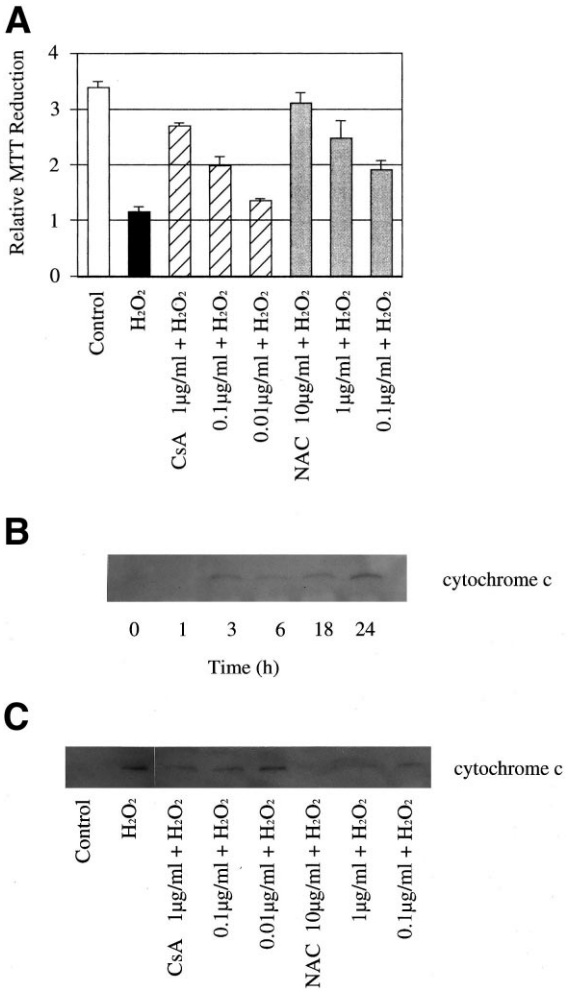

Fig. 2. Effect of CsA on H_2O_2-induced mitochondrial dysfunction. A: Mitochondrial function assayed by MTT reduction. Cells were preincubated with CsA for 10 min or NAC for 1 h before exposure to 50 μM H_2O_2. After 6 h of exposure, the cells were incubated with 2.0 μg/ml MTT for 1 h. Absorbance values were converted to MTT reduction using a standard curve generated with known numbers of viable cells. The results are presented as means ± S.D. of three independent experiments. Each experiment was done in triplicate. B: Effect of H_2O_2 on the time course of cytochrome *c* release. Cells were stimulated with 50 μM H_2O_2, and cytosolic extracts were prepared at various times after stimulation. Cytochrome *c* was detected by Western blotting using an antibody against cytochrome *c*. C: Effect of CsA on cytochrome *c* release. Cells were preincubated with CsA for 10 min or NAC for 1 h before H_2O_2 exposure. Cytosolic extracts were prepared 6 h after H_2O_2 exposure to evaluate the presence of cytochrome *c* in the cytosol.

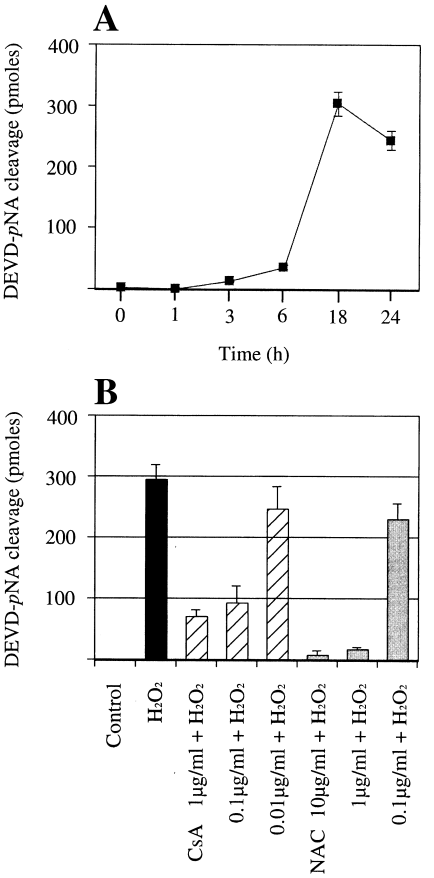

Fig. 3. A: Time course of H_2O_2-induced caspase activation in fibroblasts. B: Effect of CsA on H_2O_2-induced caspase activation. Cells were preincubated with CsA for 10 min or NAC for 1 h before exposure to 50 μM H_2O_2. After 18 h of H_2O_2 exposure, the rate of DEVD-*p*NA cleavage was measured at 405 nm. The results are presented as means ± S.D. of three independent experiments. Each experiment was done in triplicate.

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