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Immunophilin Ligands Prevent H_2O_2-Induced Apoptotic Cell Death by Increasing Glutathione Levels in Neuro 2A Neuroblastoma Cells

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We examined the effects of FK506 and its non-immunosuppressive derivative, GPI1046, on H_2O_2-induced reduction of cell viability and apoptotic cell death in Neuro 2A cells. Our results suggest that the protective properties of GPI1046 against H_2O_2-induced reduction of cell viability are equipotent with those of FK506 and may be mediated by increased intracellular concentrations of glutathione (GSH). In addition, both FK506 and GPI1046 prevented apoptotic cell death in Neuro 2A cells, although the anti-apoptotic effect of FK506 was somewhat stronger than that of GPI1046. These findings suggest that non-immunosuppressive immunophilin ligands such as GPI1046 might be potentially useful in treatment of neurodegenerative diseases without serious side effects such as immune deficiency.

Key words: hydrogen peroxide, immunophilin ligands, apoptosis, glutathione, FK506, GPI1046

It is believed that the immunosuppressive properties of immunophilin ligands do not need their neurotrophic effects so that their non-immunosuppressive derivatives retain potent neurotrophic effects (1-9) comparable to those of their mother compounds such as tacrolimus (FK506) or cyclosporin A (CsA). For instance, it has been shown that non-calcineurin (CaN) binding analogues of FK506, such as GPI1046 and V10367, which are not immunosuppressive, still promote neurite outgrowth (2, 3) and prevent cell death (10, 11). Furthermore, the neurotrophic actions of immunophilin ligands are restricted to damaged neurons, in contrast to neurotrophic factors, which also elicit neurite outgrowth in naive neurons (6). Several properties of immunophilin ligands, including their neurotrophic activities, are mediated by inhibition of rotamase activity rather than by inhibition of CaN activity (2, 3). Therefore, CaN inhibition by FK506 is probably not essential for protection against cell damage, although inhibition of both CaN and rotamase could contribute to the neuroprotective effects of FK506 (1, 3, 6, 12). We previously reported that, by increasing intracellular glutathione (GSH) levels in undifferentiated NG108-15 hybridoma cells, GPI1046 had neuroprotective effects equal to those of FK506 against H_2O_2-induced cell damage (11). Since GSH is the most abundant low-molecular-weight thiol within the cell, the GSH system has a rapid and great capacity for the prevention of reactive oxygen species (ROS)-induced cell damage and thus plays an important role in the detoxification of ROS in the brain (13, 14). However, several researchers have postulated that non-immunosuppressive immunophilin ligands do not have any beneficial effects (15-17). Therefore, it is not enough to understand that non-immunosuppressive immunophilin ligands are useful drugs for treating neurodegenerative diseases.

In the present study, we examined the neuroprotective effects of FK506 and its non-immunosuppressive derivative, GPI1046 [3-(3-pyridyl)-1-propyl (2S)-1-(3, 3-dimethyl-1, 2-dioxopentyl)-2-prolidine carboxylate], on H_2O_2-induced apoptotic cell death (18) in Neuro 2A cells which are a mouse neuroblastoma cell line. Undifferentiated NG108-15 cells mainly displays characteristics of glial lineages (19, 20), and we have already confirmed the neuroprotective effects of immunophilin ligands in undifferentiated NG108-15 cells (11). Therefore, we used neuronal cell lines to clarify the effects of immunophilin ligands on neuronal lineages. In addition, we also examined the effects of these immunophilin ligands on GSH contents in naive Neuro 2A cells.

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Materials and Methods

Neuro 2A cells were obtained from the Institute for Fermentation (IFO, Osaka, Japan), and stocks were grown using a method described previously with a minor modification (21, 22). Briefly, the cells were continuously cultured in Dulbecco's Modified Eagle Medium containing, in addition to 4 mM L-glutamine, 10% fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μg/ml) (all purchased from GIBCO BRL, Rockville, MD, USA). For experiments, cells were plated onto 96-well plates at a density of 1.6×10^4 cells/ml to assess cell viability. In addition, cells were plated onto 8-chamber slides at 1.6×10^4 cells/ml (NNI, Rochester, NY, USA) to investigate apoptotic cell death, and they were plated separately onto 6-well plates at 1.3×10^5 cells/ml to determine GSH contents. All cultures were maintained at 37 °C in a gas mixture of 5%-95% CO_2-air. Neuro 2A cells were grown for 4-5 days and then used in the present studies.

In the assessment of cell viability, cells were treated with immunophilin ligands, FK506 (Fujisawa, Osaka, Japan) or GPI1046, at a final concentration of 1, 10 or 100 nM, to evaluate their protective effects against H_2O_2-induced cell damage. Following a 24 h-incubation after the treatment with immunophilin ligands, the cell culture medium was replaced with a fresh medium, and then cells were treated with H_2O_2 at a final concentration of 300 μM. Following a 24 h-incubation after H_2O_2 challenge, cell viability was determined by a 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST-1) cell counting kit (Dojin Lab, Kumamoto, Japan). FK506 or GPI1046 was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was maintained at $\leq$ 0.1%. The cell viability in each well of the 96-well plate was determined by quantitative colorimetric assay with WST-1 (11, 23), a modification of the standard MTT assay. Viability was expressed as a percentage of the cell viability from each 0.1% DMSO-treated control culture in the non-H_2O_2 exposed group.

In addition, the morphological changes in the cells were observed by fluorescence microscopy after staining with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) or propidium iodine (PI; Molecular Probes, Eugene, OR, USA). Cells were fixed with 4% paraformaldehyde (in 0.1 M phosphate buffer) for 10 min. After washing with 10 mM phosphate-buffered saline (PBS), Hoechst 33342 (20 μg/ml) or PI (4 μg/ml) was added to the cells for 3 min. Slides were rinsed briefly with PBS, air-dried, and then mounted in anti-fluorescein fading medium (PermaFluor; Lipshaw Co., Pittsburgh, PA, USA). After staining with Hoechst 33342 (emission, above 420 nm; excitation, 330-385 nm) or PI (emission, above 580 nm; excitation, 520-550 nm), cells were assessed as having undergone apoptosis or necrotic cell death by fluorescence microscopy.

GSH levels were determined using the enzymatic recycling method of Tietze (24) with some modifications. For preparation of the GSH sample, Neuro 2A cells were grown for 24 h in the presence or absence of immunophilin ligands. Cells were homogenized in 0.1 M phosphate buffer (pH 7.4) and then were added at equivalent volumes to 10% trichloroacetic acid and cellular homogenates. Acid extracts were mixed with 0.01 M phosphate buffer (pH 7.4, 580 μl), NADPH (4 mM, 50 μl) and glutathione reductase (6 U/ml, 100 μl), and incubated for 5 min at 37 °C. 3-Carboxy-4-nitrophenyl disulfide (10 mM, 50 μl) was added just before reading absorbance. The formation of 2-nitro-5-thiobenzoic acid was measured at 450 nm for 6 min. Total GSH was determined from a standard curve constructed using known amounts of GSH.

Data are presented as mean $\pm$ SEM. Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnett's test ($P < 0.05$).

Results

We examined the protective effects of immunophilin ligands against H_2O_2-induced cell damage. Exposure to H_2O_2 (300 μM) for 24 h reduced cell viability to 51.4 $\pm$ 2.3% in 0.1% DMSO-treated Neuro 2A cells (Fig. 1). However, pretreatment with FK506 and GPI1046 increased cell viability in a dose-dependent manner (Fig. 1). In particular, a high dose of both FK506 and GPI1046 (100 nM) significantly improved H_2O_2-induced reduction of cell viability in contrast to that of the 0.1% DMSO-treated group (Fig. 1). In addition, the occurrence of apoptosis or necrotic cell death were differentiated by morphological observation after staining with Hoechst 33342 or PI. By means of fluorescence microscopy, Neuro 2A cells positively stained with Hoechst 33342 showed condensed and fragmented nuclei typical of H_2O_2-induced apoptotic cell death in the 0.1% DMSO-

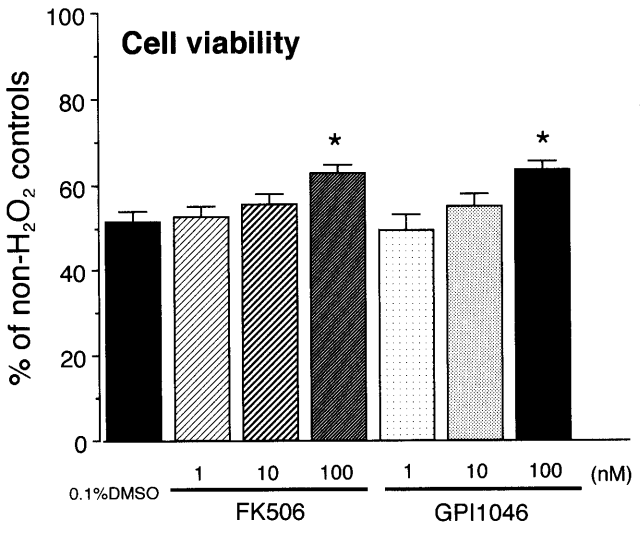

Fig. 1 Viability of Neuro 2A cells was measured by WST-1 colorimetric assay. Neuro 2A cells were maintained in the presence or absence of FK506 or GPI1046 (final concentrations of 1, 10, 100 nM) for 24 h and then of 300 μM of H_2O_2. After an additional 24 h, cell damage was assessed by WST-1 assay. Each value is the mean ± SEM (n = 12) expressed as a percentage of control cultures treated with autoclaved water instead of H_2O_2. *$P < 0.05$ compared with 0.1% DMSO-treated control cultures in non-H_2O_2 exposed group.

treated group (Fig. 2). However, both FK506 and GPI1046 (100 nM) prevented H_2O_2-induced apoptotic cell death, although the anti-apoptotic effect of FK506 was somewhat stronger than that of GPI1046 (Fig. 2). On the other hand, H_2O_2 did not induce necrotic cell death since PI staining was not investigated in any experimental groups (data not shown).

To clarify the mechanism of the protective effects of immunophilin ligands, we determined the effects of FK506 and GPI1046 on GSH contents in naive Neuro 2A cells. Treatment with FK506 (100 nM) or GPI1046 (100 nM) significantly increased GSH contents to 180.7% or 169.6%, respectively, compared with 0.1% DMSO treatment (Fig. 3).

Discussion

Oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and stroke, due to the ROS (25–27). Therefore, antioxidant properties may contribute to the neuroprotective effects of drugs on neurodegenerative diseases. To control the harmful effects of oxidative stress, efficient detoxification pathways have been identified in various cells. Of the various antioxidant systems in the brain, the GSH system is particularly important in controlling cellular redox states and is the primary defense mechanism for removal of peroxide from the brain (13, 14, 28). Furthermore, cellular GSH has been found to be important in modulating oxidative stress-induced apoptosis in several cell lines (18, 29–31). Moreover, apoptosis has been prevented by increasing intracellular GSH levels in several models of apoptosis (29–31). In the case of neuronal lineages such as neuroblastoma or neurons, GSH has also protected against oxidative stress-induced apoptotic cell death (32–35). We have reported previously that the increasing effects of GPI1046 on the level of intracellular GSH are equipotent to those of FK506 in undifferentiated NG108-15 cells (11). In the present study, we also demonstrated that both GPI1046 and FK506 enhanced intracellular GSH contents in Neuro 2A cells. Furthermore, the efficacy of FK506 and GPI1046 for Neuro 2A cells (neuronal lineages) was almost equipotent to that for undifferentiated NG108-15 cells (glial lineages). In addition, both FK506 and GPI1046 had neuroprotective properties against H_2O_2-induced reduction of cell viability and apoptotic cell death. However, the anti-apoptotic effect of FK506 was somewhat stronger than that of GPI1046, although the neuroprotective effect of GPI1046 on H_2O_2-induced reduction of cell viability was similar to that of FK506. Nevertheless, our present and previous findings (11) are almost in agreement with those of Steiner's previous studies (3, 4, 6, 8).

On the other hand, CaN has been implicated in the signal transduction pathway leading to Ca^{2+}-dependent apoptosis (36). Recent studies have shown that anti-CD3-induced apoptosis is mediated by Fas-ligand production, which is regulated by the CaN-activated nuclear factor of activated T-cells (NF-AT) (37) and the interaction between Fas and Fas-ligand (38–40). CaN has also been found to be activated by the dephosphorylation of Bad and nitric oxide synthase (41, 42). Furthermore, CaN-induced apoptosis is due to activation of the cytochrome c/caspase-3 pathway in neurons (43). Therefore, CaN appears to participate in apoptosis in various ways, and inhibition of CaN activity may be important for neuroprotective properties of immunosuppressive immunophilin ligands, such as FK506, against CaN-related apoptosis (36, 44, 45). Therefore, our results indicate

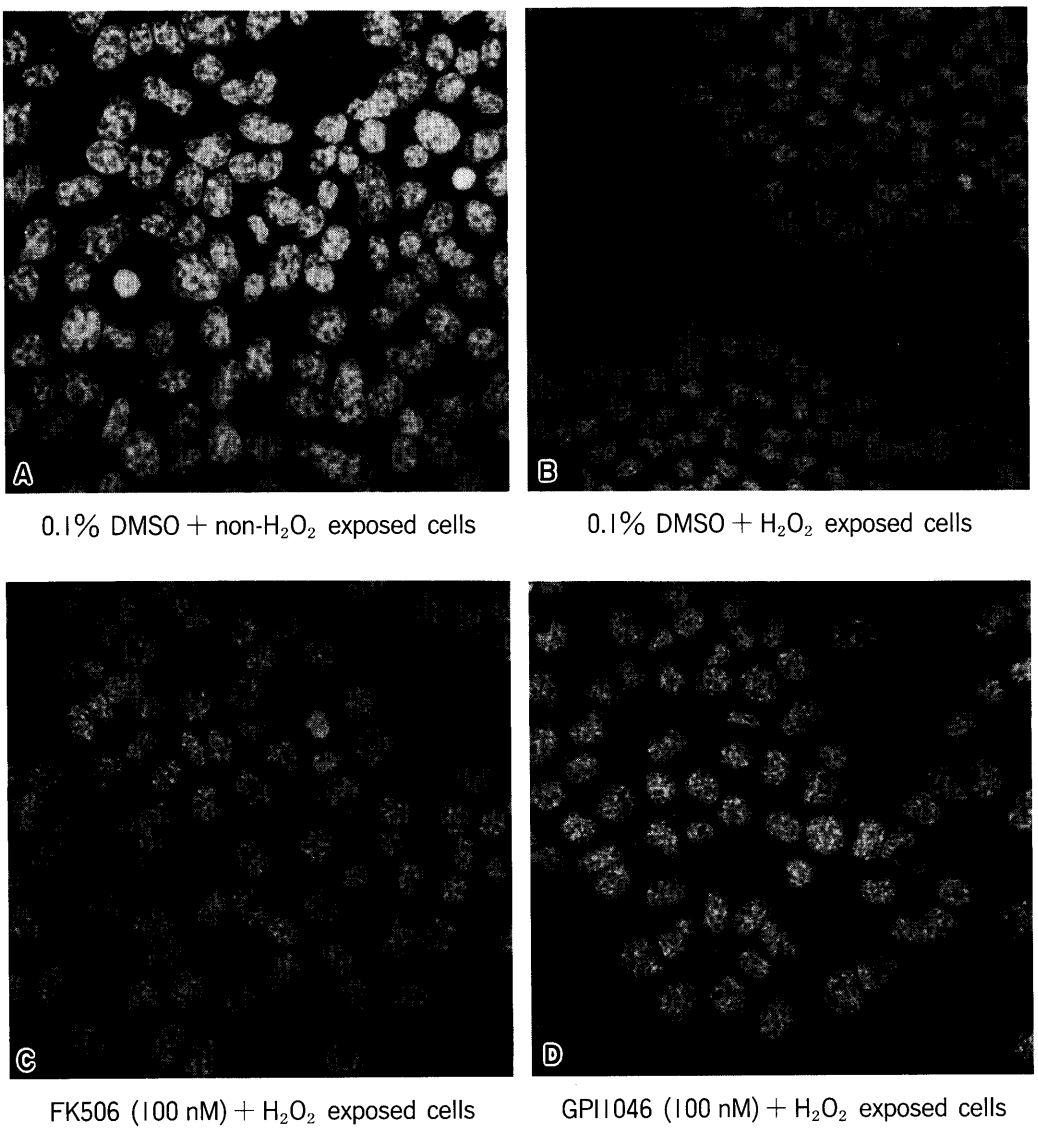

Fig. 2 H_2O_2 (300 μM)-induced apoptotic cell death in Neuro 2A cells with Hoechst 33342 staining. Neuro 2A cells were maintained in the presence or absence of FK506 or GPI1046 (final concentrations of 100 nM) for 24 h and then of 300 μM of H_2O_2 for 24 h. Panel **A**, 0.1% DMSO-treated + non-H_2O_2 exposed group; Panel **B**, 0.1% DMSO-treated + H_2O_2 exposed group; Panel **C**, FK506 (100 nM)-treated + H_2O_2 exposed group; Panel **D**, GPI1046 (100 nM)-treated + H_2O_2 exposed group.

that H_2O_2-induced apoptotic cell death in Neuro 2A cells may be dependent not only on the GSH depletion pathway but also the CaN pathway.

In conclusion, our present results suggest that the cellular protective properties of GPI1046, a non-immunosuppressive FK506 derivative, is equipotent to that of FK506 and may be mediated by increasing intracellular GSH levels. Moreover, both FK506 and GPI1046 prevented apoptotic cell death in Neuro 2A cells, although the anti-apoptotic effect of FK506 was somewhat stronger than that of GPI1046. Thus, non-immunosuppressive immunophilin ligands, such as GPI1046, might have a potentially beneficial effect on neurodegenerative diseases, particularly since they do not cause serious side effects such as immune deficiency. In addition, the GSH-activated effect could be regarded as a beneficial property of neuroprotective drugs.

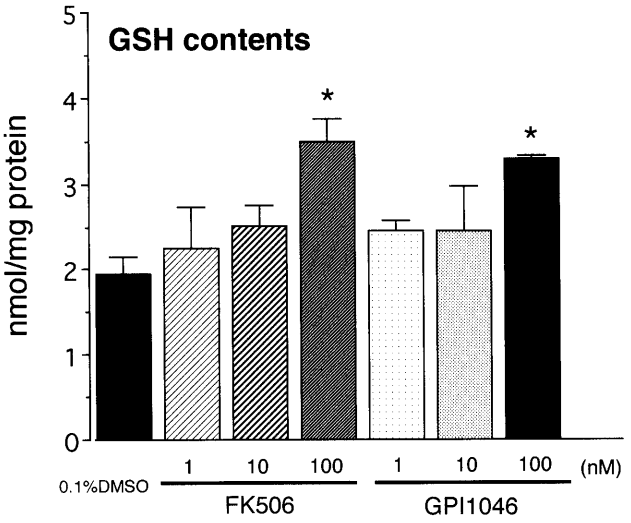

Fig. 3 Effects of FK506 and GPI1046 on GSH contents in Neuro 2A cells. Neuro 2A cells were grown for 24 h in the presence or absence of FK506 or GPI1046 (1, 10, 100 nM). Each bar represents the mean ± SEM (n = 6). $*P < 0.05$ compared with 0.1% DMSO-treated control cultures.

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