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Protective effect of FK506 against apoptosis of SH-SY5Y cells correlates with regulation of the serum inducible kinase gene

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Abstract

Recently, we established an in vitro model of apoptosis induced by exposure of neuroblastoma SH-SY5Y cells to thapsigargin, an endoplasmic reticular calcium-ATPase inhibitor, and demonstrated that FK506 (tacrolimus) protected against apoptosis. The purpose of this paper was to investigate a possible correlation between the protective effect of FK506 against apoptosis and the regulation of the serum inducible kinase (SNK) and fibroblast growth factor inducible kinase (FNK) genes—which are polo-like kinases expressed abundantly in the brain by FK506. Thapsigargin increased the mRNA level of SNK and FNK in SH-SY5Y cells. FK506 inhibited the increase in SNK mRNA but not FNK mRNA. Deletion analysis of the SNK promoter showed that the promoter site, which was regulated by thapsigargin and FK506 in a calcineurin-dependent manner, is a cAMP response element (CRE)/activating transcription factor (ATF)-like element located 84 base pairs (bp) proximal to the transcriptional initiation site. Although transcription of the SNK gene was also regulated by tunicamycin, etoposide, or staurosporine, FK506 did not show any effects on these regulations. We recently reported that FK506 did not protect against apoptosis induced by these agents. These results indicate that the induction of SNK mRNA by thapsigargin in SH-SY5Y cells is regulated by FK506 via an inhibition of calcineurin at the transcriptional stage, and the transcriptional regulation of the SNK gene by FK506 was well correlated with the protective effect of the compound against apoptosis. Thus, transcriptional regulation of the SNK gene may be a biological marker for analysis of apoptosis of SH-SY5Y cells.

Keywords: FK506; Thapsigargin; Apoptosis; SH-SY5Y cells; Serum inducible kinase; Biological marker

1. Introduction

FK506 (tacrolimus) and cyclosporine A are clinically effective immunosuppressive drugs which are widely used to inhibit allograft rejection [1]. FK506 is known to exert its immunosuppressive effects by inhibiting the dephosphorylation induced by calcineurin, a calcium-activated protein phosphatase, after binding to intracellular proteins referred to as FK506-binding proteins (FKBPs) [2]. Furthermore, FK506 has been reported to show potent neuroprotective effects in animal models, such as those of stroke and neurodegenerative diseases [3–7]. However, the mechanism of the neuroprotective effect of FK506 is not fully understood. Recently, we established an in vitro model of apoptosis induced by exposure of neuroblastoma SH-SY5Y cells to thapsigargin, an endoplasmic reticular calcium-ATPase inhibitor, and demonstrated that FK506 protected against the apoptosis [8]. On the other hand, FK506 did not show any protection against apoptosis induced by other agents, such as tunicamycin, etoposide, and staurosporine. Although we provided the detailed pharmacological profiles related to the neuroprotective effects of FK506 in our previous paper, we did not discuss the molecular mechanism.

Serum inducible kinase (SNK), also known as polo-like kinase 2 (Plk2), is a member of the 'polo' family of serine-threonine protein kinases, which play a role in the normal cell cycle [9]. The founding member of this kinase family, polo, was initially identified in *Drosophila melanogaster* as a gene required for mitotic progression [10]. In mammalian

Abbreviations: FK506, the immunosuppressive macrolactamlactone tacrolimus; SNK, serum inducible kinase; FNK, fibroblast growth factor inducible kinase; Plk, polo-like kinase; NF-IL3, nuclear factor interleukin-3; CRE, cAMP response element; CREB, cAMP response element binding protein; ATF, activating transcription factor

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cells, two other family members have been identified: polo-like kinase 1 (Plk1) and fibroblast growth factor inducible kinase (FNK), also known as Polo-like kinase 3 (Plk3) [11,12]. The polo-like kinase family is characterized by a conserved N-terminal kinase domain and the presence of a highly conserved domain of 28 amino acid residues in the C-terminus, referred to as the polo box [13,14]. Two polo-like kinases, SNK and FNK, were originally identified as early immediate genes [9,15]. It has been reported that SNK and FNK, but not Plk1, are abundantly expressed in the rat brain, and that the expression of these genes is regulated by neuronal activations such as those by seizure or long-term potentiation [16]. Recent studies have demonstrated that ectopically expressed SNK and FNK induced chromatin condensation and apoptosis [17–19]. On the contrary, it has also been reported that SNK protects against apoptosis. SNK is a novel target gene of p53, which is known as an anti-apoptosis gene, and small interfering RNA-mediated SNK silencing leads to apoptosis during mitosis after treatment with antimicrotubule agents [20]. Thus, the role of the SNK gene in apoptosis remains elusive. These reports led us to investigate the involvement of the SNK and FNK genes in the anti-apoptotic effect of FK506.

In this study, we investigated the possible correlation between the protective action of FK506 against apoptosis and the regulation of the SNK and FNK genes by FK506. The treatment of SH-SY5Y cells with thapsigargin increased both the SNK and FNK mRNA levels. FK506 prevented the increase in the SNK mRNA level but not that in the FNK mRNA level. We performed a detailed promoter analysis of the SNK gene and identified a promoter site regulated by thapsigargin and FK506. Moreover, in order to define a correlation between the suppressive effect of FK506 against apoptosis and the regulation of the SNK gene by FK506, we examined the effects of FK506 on the regulation of the SNK gene caused by stimuli other than thapsigargin, all of which were shown to induce apoptosis on which FK506 showed no protective effects.

2. Materials and methods

2.1. Reagents

Thapsigargin was purchased from Wako Pure Chemicals. Cyclosporin A, rapamycin, tunicamycin, etoposide, staurosporine, and forskolin were purchased from Sigma. FK506 was generated at Fujisawa Pharmaceuticals.

2.2. Cell cultures

Human neuroblastoma SH-SY5Y cells were obtained from the European Collection of Animal Cell Cultures. SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma) supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of total RNA

SH-SY5Y cells were seeded at 1×10^6 cells/well in 2 ml of D-MEM on plastic six-well plates and allowed to grow for 24 h. The medium was replaced with serum-free medium and the cells were pretreated for 2 h with the indicated concentrations of FK506, cyclosporin A, or vehicle. The cells were then stimulated with 100 nM thapsigargin or vehicle for 24 h. At the indicated time points after the addition of thapsigargin or vehicle, total RNA was isolated from these cells with TRIzol reagent (Life Technologies). Total RNA of three independent experiments were prepared for each group ($n = 3$).

2.4. Real-time quantitative RT-PCR

The mRNA levels of SNK, FNK, nuclear factor interleukin-3 (NF-IL3), and elongation factor 1-alpha were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using a PE Applied Biosystems prism model 7700 sequence detection instrument (Applied Biosystems). Pair-wise primers and probes were designed to detect specifically human SNK, FNK, NF-IL3, and EF1-alpha genes, respectively, using primer Express software (Applied Biosystems). The sequences of the PCR primers and probes are listed in Table 1. Experiments were performed with TaqMan EZ RT PCR CORE REAGENT (Applied Biosystems) according to the manufacturer's protocol. The amplification mixtures (25 μl) contained 62.5 ng of total RNA, 300 μM dATP, dCTP, and dGTP, 600 μM dUTP, 4 mM $Mg(OAc)_2$, 5 units of r*Tth* DNA polymerase, 0.5 units of AmpErase uracil *N*-glyco-

Table 1
Sequences of primers and probes for real-time PCR

Human SNK	
Sense	5′-GTCAGAGGGACTCTTGGCAG-3′
Antisense	5′-GCAACACTTCCCATGGTACTG-3′
Probe	5′-6FAM-TAGCAGCAGCAGTGAATGC-CTTGAAG-TAMRA-3′
Human FNK	
Sense	5′-GCTTCTCCAATAAGTTCGGC-3′
Antisense	5′-GCCATCGTTGAAGAGCACA-3′
Probe	5′-6FAM-CTGTCCAGCCGCCGTGTGG-TAMRA-3′
Human NF-IL3	
Sense	5′-GGTGTGGTAGGAAAGTCATCTGA-3′
Antisense	5′-TCAACTGGAGAATGGATGGG-3′
Probe	5′-6FAM-AGAAGACGAGCAACAGGT-CCCCAA-TAMRA-3′
Human EF1-alpha	
Sense	5′-TAAGGATGGCAATGCCAGT-3′
Antisense	5′-TTGGACGAGTTGGTGGTAGG-3′
Probe	5′-6FAM-CACGCTGCTTGAGGCTCT-GGAC-TAMRA-3′