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Transcriptional Regulation of Human GD3 Synthase (hST8Sia I) by Fenretinide in Human Neuroblastoma SH-SY-5Y Cells

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To elucidate the mechanism underlying the regulation of hST8Sia I gene expression in FenR-induced SH-SY5Y cells, we characterized the promoter region of the hST8Sia I gene. Functional analysis of the 5′-flanking region of the hST8Sia I gene showed that the -1146 to -646 region functions as the FenR-inducible promoter of hST8Sia I in SH-SY5Y cells. Site-directed mutagenesis indicated that the NF-&B binding site at -731 to -722 was crucial for the FenR-induced expression of hST8Sia I in SH-SY5Y cells. To investigate which signal transduction pathway was involved in FenR-stimulated induction of hST8Sia I in SH-SY5Y cells, we performed Western blot analysis using phospho-specific antibodies in order to measure their degree of regulatory phosphorylation. Phosphorylations of AKT and RelA (p65) subunit of NF-κB were significantly elevated in cytosolic and nuclear fractions of FenR-stimulated SH-SY5Y cells, respectively, than in control or DMSO-treated SH-SY5Y cells. These results suggest that FenR induce transcriptional up-regulation of hST8Sia I gene expression through translocation of RelA (p65) subunit of NF-κB to nucleus by AKT signal pathway in SH-SY5Y cells.

Key words : Human GD3 synthase, fenretinide, transcription factor, SH-SY5Y

Introduction

Gangliosides are the sialic acid (NeuAc)-containing glycosphingolipids existing as component molecules of the outer leaflet of the plasma membrane of vertebrate cells [15]. They play important roles in a variety of biological processes, such as cell-cell interaction, adhesion, cell differentiation, growth control, oncogenesis, metastasis, and receptor function [5,6]. Among these gangliosides, it is known that the disialoganglioside GD3 is weakly expressed in most normal tissues, but highly expressed during development and in pathological conditions such as cancer, neurodegenerative disorders and atherosclerosis [13]. It was also reported that GD3 is responsible for diverse events such as proliferation, differentiation, cellular ageing and apoptosis [14].

Fenretinide (FenR), a synthetic analogue of retinoic acid, induces apoptosis of neuroblastoma cells and is currently used in chemoprevention clinical trials for neuroblastoma and other cancers [4,12]. A previous study has shown that and reactive oxygen species (ROS) generation and apoptosis in human neuroblastoma SH-SY-5Y cells are associated with increased levels of ceramide and glucosylceramide (GlcCer) synthase activity, GD3 synthase (ST8Sia I) activity, and GD3 by FenR treatment [10]. These data indicate that FenR promotes ceramide and ultimately GD3 productions through pathway including sphingomyelin hydrolysis and ST8Sia I activity in SH-SY-5Y cells. Despite increasing understanding of the important physiological roles for GD3 and ST8Sia I in FenR-induced apoptotic process, however, little is yet known about how the ST8Sia I gene expression responsible for GD3 formation in FenR-induced apoptosis is regulated.

In this study, we investigated the transcriptional regulation mechanism of human GD3 synthase (hST8Sia I) gene expression in response to FenR signaling. The present results clearly indicate that hST8Sia I gene expression is up-regulated especially through NF-κB-dependent activation in FenR-induced SH-SY-5Y cells.

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Materials and Methods

Cell cultures

The human neuroblastoma cell line SH-SY-5Y, obtained from American Type Culture Collection (Manassas, VA, USA) was maintained at 37°C in a 5% CO_2 incubator and cultured in in a 1:1 mixture of DMEM and Ham F12 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Life Technologies; Grand Island, NY, USA). FenR (Sigma Chemical Co., USA) was dissolved in dimethyl sulfoxide

Table 1. Sequence of the primers used in this study

Primer name	Sequence	Strand
GlcCer-F	5′-GCTGACCGAGGTTGGAGGTT-3′ -3′	Sense
GlcCer-R	5′-CACAGTGTGCCACCCTGGAC-3′	Antisense
NSMase-F	5′-CAGTTCGTGCCAAAACAGCCA-3′	Sense
NSMase-R	5′-ACTTAAATGTGCCGCAATGTCCC-3′	Antisense
ASMase-F	5′-CAGGGTTCCTGGCTGGGCAGCA-3′	Sense
ASMase-R	5′-GGTCCTGGACCATGAGACCTAC-3′	Antisense
hST3Gal V-F	5′-CCCTGCCATTCTGGGTACGAC-3′	Sense
hST3Gal V-R	5′-CACGATCAATGCCTCCACTGAGATC-3′	Antisense
hST8Sia I-F	5′-TGTGGTCCAGAAAGACATTTGTGGACA-3′	Sense
hST8Sia I-R	5′-TGGAGTGAGGTATCTTCACATGGGTCC-3′	Antisense
β-actin-F	5′-CAAGAGATGGCCACGGCTGCT-3′	Sense
β-actin-R	5′-TCCTTCTGCATCCTGTCGGCA-3′	Antisense

(DMSO) at stock concentration of 10 mM. To induce expression of the hST8Sia I gene with FenR, SH-SY-5Y cells were cultured for various time periods in the presence of 6 μM FenR.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SH-SY-5Y cells using Trizol reagent (Invitrogen, USA). Two micrograms of RNA was subjected to reverse transcription with random nonamers using Takara RNA PCR kit (Takara, Japan) according to the manufacturer's protocol. The resulting cDNA was amplified by PCR with the corresponding primers shown in Table 1. PCR products were subcloned into pGEM-T vector (Promega, USA) and then sequenced. These genes were found to be identical to the expected cDNA.

Transfection and luciferase assay

The luciferase reporter plasmids used herein, namely pGL3-2646/-646 and its derivatives (pGL3-1146/-646 to pGL3-2246/-646) with base substitutions in the CREB, AP-1, c-Ets-1, NF-κB binding sites, have been described elsewhere [7-11]. To analyze hST8Sia I promoter activity in response to FenR treatment, SH-SY-5Y cells (3.0×10^5 cells/well) were seeded in 24-well tissue culture plates and allowed to grow to 70% confluence, at which point they were transiently co-transfected with 0.5 μg of the indicated reporter plasmid and 50 ng of the control *Renilla* luciferase vector pRL-TK (Promega; Madison, WI, USA), using 1 μl Lipofectamine 2000 (Invitrogen). After a 12 hr recovery in normal medium without FenR, the medium was changed to medium containing 6 μM FenR and incubated for an additional 12 hr, after which cells were collected and treated with passive lysis buffer (Promega). Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, and a GloMax^{TM} 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to *Renilla* luciferase activity and expressed as a fold induction over the empty pGL3-Basic vector, used as a negative control. Independent triplicate experiments were performed for each plasmid.

Western blot analysis

SH-SY-5Y cells treated with 6 μM FenR for 12 hr were lysed in cold RIPA buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktail. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, USA). Lysates and nuclear extracted proteins were prepared by four cycles of freezing and thawing of the harvested cells followed by centrifugation. Twenty μg of 6 samples of total cell lysates were size fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using the Hoefer electrotransfer system (Amersharm Biosciences, UK). Membranes were incubated with phospho-ERK1/2, phospho-JNK p46/p54, Jun D p35/p40 and NF-κB p50/p65 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and phospho-AKT (Ser473), and phospho-CREB (Ser133) purchased from Cell Signaling Technology (Beverly, MA, USA), and GAPDH and anti-lamine A/C antibodies purchased from Millipore Corporation (Temecula, CA, USA). Detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody and anti-rabbit the chemiluminescence system

(Amersharm Biosciences, UK).

Results

Effect of FenR on mRNA expression levels of genes involved in ceramide generation and metabolism in SH-SY5Y cells

It is known that FenR enhances intracellular levels of the lipid secondary messenger ceramide during apoptosis and intracellular ceramide levels can increase as a result of hydrolysis of membrane sphingomyelin by neutral sphingomyelinase (NSMase) and acid sphingomyelinase (ASMase) [4,10,12]. It was also reported that increased intracellular ceramide levels result from sphingomyelin hydrolysis by ASMase during FenR-induced apoptosis of SH-SY5Y cells [10]. Ceramide is metabolized to the ganglioside GD3 via glucosylceramide synthase (GlcCer synthase), GM3 synthase (ST3Gal V) and ST8Sia I [11,12].

To investigate the effect of FenR on mRNA expression levels of genes involved in ceramide generation and metabolism in SH-SY5Y cells, after cells were treated for 12 hr with various concentration of FenR, mRNA levels of ASMase, NSMase, GlcCer synthase, hST3Gal V, and hST8Sia I were analyzed by RT-PCR. As shown in Fig. 1, the mRNA levels of NSMase and hST8Sia I gene expression increased by FenR treatment in dose-dependent manner, whereas ASMase, GlcCer synthase, and hST3Gal V mRNA expressions were not affected by FenR. On the basis of this result, to investigate further whether FenR stimulation induces up-regulation of the hST8Sia I gene in SH-SY5Y cells, we treated the cells with 6 μM FenR for varying periods of time, and we examined the expression level of hST8Sia I mRNA using RT-PCR. As shown in Fig. 2, time-dependence increase of hST8Sia I mRNA expression was detected, which peaked in 9 hr after FenR treatment and then significantly decreased at 24 hr. These results clearly show that the expression of hST8Sia I was up-regulated by FenR. Because FenR is well known to induce apoptosis in a variety of cancer cells including human neuroblastoma [4,11], its effects on the induction of apoptosis in SH-SY5Y cells were examined. Massive apoptotic cells, showing cell blebbing, shrinkage and nuclear fragmentations, were observed under the phase contrast microscopy in FenR-treated cells (data not shown).

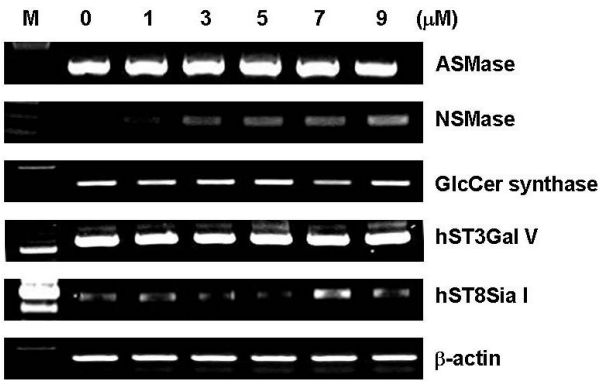

Fig. 1. RT-PCR analysis of mRNA expression levels of genes involved in ceramide generation and metabolism in FenR-treated SH-SY5Y cells. Total RNA from SH-SY5Y cells was isolated after FenR treatment for 24 hr at different concentrations (0, 1, 3, 5, 7, 9 μM) and mRNAs were detected by RT-PCR. As an internal control, parallel reactions were performed to measure levels of the housekeeping gene β-actin. ASMase, acidic sphingomyelinase; NSMase, neutral sphingomyelinase; GlcCer synthase, glucosylceramide synthase.

Analysis of transcriptional activity of hST8Sia I promoter by FenR in SH-SY5Y cells

To determine whether the 5′-flanking sequence of the hST8Sia I gene contained a FenR-responsive promoter, we

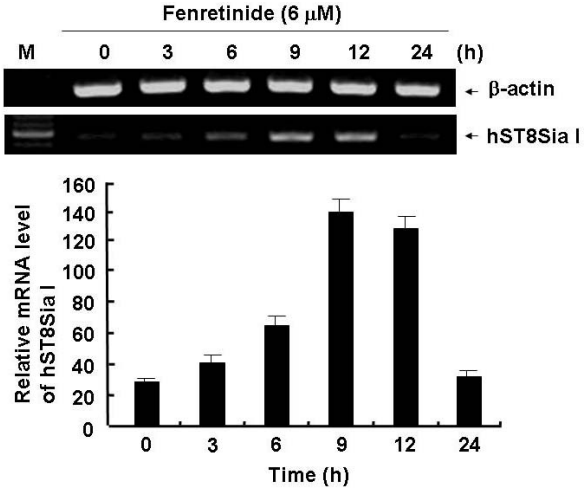

Fig. 2. RT-PCR analysis of hST8SiaI mRNA expression levels in FenR-treated SH-SY5Y cells. Total RNA from SH-SY5Y cells was isolated after 6 μM FenR treatment at indicated time and hST8Sia I mRNA was detected by RT-PCR. As an internal control, parallel reactions were performed to measure levels of the housekeeping gene β-actin. The densitometric intensity of hST8Sia I band was shown in under panel. Data represent the relative values±SD of three independent experiments and the mean values from each experiment were compared using ANOVA followed by Duncan's tests. Values not sharing the same letter are significantly different from one another (p<0.05).

prepared luciferase constructs carrying serial 5′deletions of the hST8Sia I promoter, transfected them into SH-SY5Y cells, and then treated the transfected cells with FenR. We monitored the subsequent expression of the luciferase reporter gene using the dual-luciferase reporter assay system, after which we measured luciferase activity with a luminometer. As shown in Fig. 3, cells harboring the pGL3-1146/-646 construct showed a remarkable increase in luciferase activity after FenR treatment, about two-fold higher than untreated transfected cells. In contrast, FenR stimulation did not alter the luciferase activity in cells expressing the pGL3-basic (negative control) or other 5′-deleted hST8Sia I promoter constructs. These results clearly suggest that the region containing nucleotides -1146 to -646 played an important role in the expression of hST8Sia I and its functions as the FenR-inducible promoter in SH-SY5Y cells.

Identification of FenR-responsive element in nucleotide -1146 to -646 region of hST8Sia I promoter

We have previously demonstrated that the region from -1146 to -646 contained putative binding sites such as c-Ets-1, AP-1, CREB and NF-κB binding sites [7-9]. To determine whether these binding sites contributed to FenR-induced expression of hST8Sia I in SH-SY5Y cells, four mutants (pGL3-1146/-646mtCREB, mtAP-1, mtNF-κB and mtc-Ets-1) were used, which contained the exact same construct as wild type pGL3-1146/-646 except that combined nucleotides within these binding sites had been changed [7]. A series of substituted mutations of luciferase constructs (Fig. 4) were transfected into SH-SY5Y cells and luciferase assays were carried out. The activity of each construct was compared to that of pGL3-basic and wild type (pGL3-1146/-646) as negative and positive controls, respectively. In FenR-treated cells, pGL3-1146/-646mtNF-κB of four constructed mutations markedly reduced transcriptional activity to more than four-fold of pGL3-1146/-646wt, whereas the activities of the pGL3-1146/-646mtCREB, mtAP-1 and mtc-Ets-1 constructs were not decreased (Fig. 4). These results indicate that this NF-κB site is crucial for the FenR-induced expression of hST8Sia I in SH-SY5Y cells.

Analysis of signaling pathway involved in hST8Sia I gene expression in FenR-stimulated SH-SY5Y cells

To investigate the mechanism underlying transcriptional activation of hST8Sia I in FenR-stimulated SH-SY5Y cells, by Western blot with the samples divided into cytosolic and nuclear fractions we analyzed the change of protein levels involved in signaling pathway in FenR-stimulated SH-SY5Y cells. In cytosolic fraction, as shown in Fig. 5, the protein level of phospho-AKT was significantly increased by FenR treatment, compared to control or DMSO treatment, whereas phospho-ERK1/2 and phospho-JNK p54/p46 unchanged by FenR stimulation in SH-SY5Y cells. In nuclear faction, the protein level of NF-κB p65 was apparently increased by FenR stimulation, whereas phospho-CREB, Jun D p35/p40, and NF-κB p50 showed the same results as control or DMSO

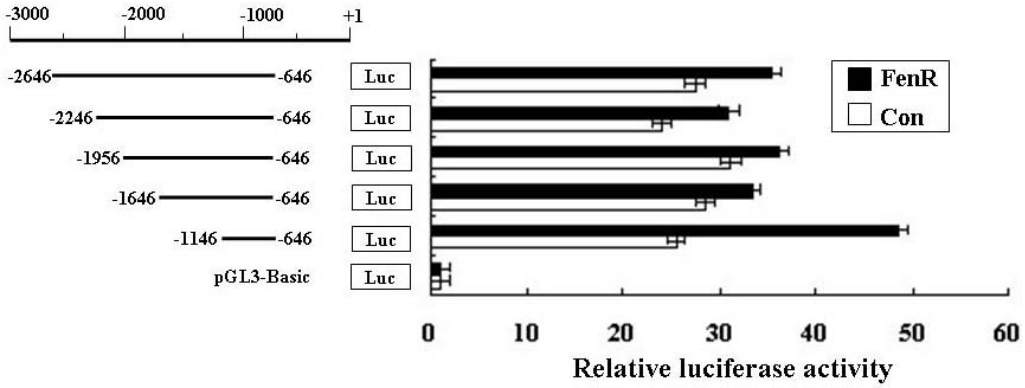

Fig. 3. Deletion analysis of hST8Sia I promoter in SH-SY5Y cells before and after FenR treatment. A schematic representation of DNA constructions containing three equal lengths from different starts of the 5′-flanking region of hST8SiaI gene linked to the luciferase reporter gene is presented. The length sizes are shown and the translation start site is indicated as +1. pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into SH-SY5Y cells with pRL-TK as an internal control. The transfected cells were incubated in the presence (solid bar) or absence (open bar) of 6 μM FenR for 24 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the means±SD of three independent experiments with triplicate measurements.

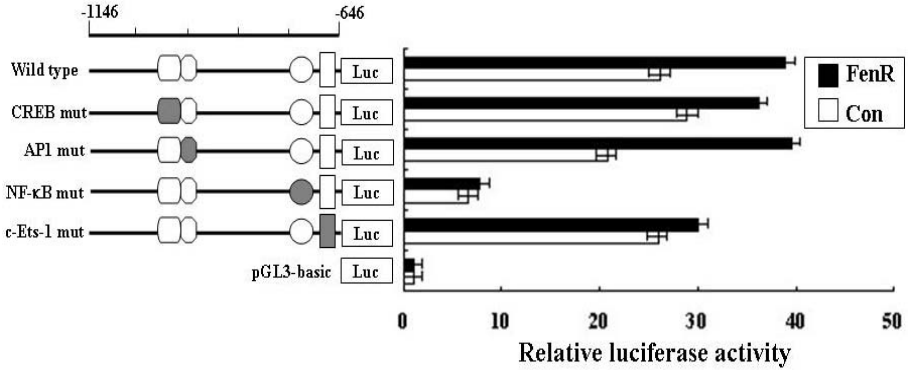

Fig. 4. Mutation promoter assay for the transcription factor binding sites in hST8Sia I gene. pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into SH-SY5Y cells with pRL-TK as an internal control. The transfected cells were incubated in the presence (solid bar) or absence (open bar) of 6 μM FenR for 24 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the means±SD of three independent experiments with triplicate measurements. The mutation mark of promoter construction is indicated by closed form or opened form (wile-type).

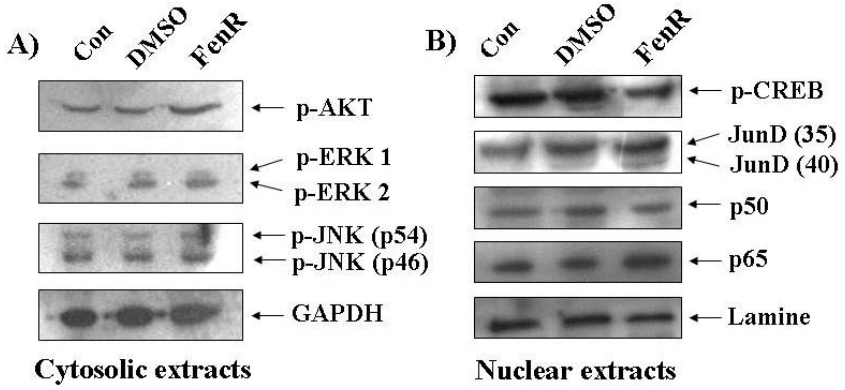

Fig. 5. Nucleic translocation of the activated NF-κB protein via AKT pathway in FenR-induced SH-SY5Y cells. Cytosolic protein fraction (A) and the nuclear protein fraction (B) from SH-SY-5Y cells were isolated after DMSO or 6 μM FenR_treatment for 24 h. The fractional protein lysates from these cells were prepared with different method. The expression or phosphorylation change for the isolated fractional proteins was detected by Western blot analysis using the specific antibodies for phospho-AKT, phospho-ERK, phospho-JNK, phospho-CREB, JunD and NF-κB p50/p65. GAPDH and lamine were used as control of cytosolic and nuclear proteins, respectively.

treatment. This result indicates that only RelA (p65) subunit of NF-κB was translocated to the nucleus by FenR stimulation through AKT signaling pathway in SH-SY5Y cells.

Discussion

Although FenR-induced apoptosis of SH-SY5Y cells is known to be mediated by ASMase, GlcCer synthase, and ST8Sia I [4,10-12], their mRNA expression levels have not reported yet. In this study, we have shown for the first time mRNA expression levels of genes involved in ceramide generation and metabolism in FenR-stimulated SH-SY5Y cells. Previous study has shown that ceramide generation in response to FenR in SH-SY5Y cells is mediated by ASMase, but not NSMase. Contrary to this finding, however, our present result revealed that the mRNA expression levels of NSMase gene with hST8Sia I increased by FenR treatment in dose-dependent manner, whereas mRNA levels of ASMase with GlcCer synthase and hST3Gal V mRNA expressions were not affected by FenR. This result suggests that activities of these two enzymes are different from their transcription levels. In the present study, we have also elucidated a part of the transcriptional regulation mechanism that underlies hST8Sia I gene induction in response to FenR. In

order to investigate FenR-responsive elements involved in the increased expression of the hST8Sia I gene in SH-SY5Y cells, we first tried to identify the region within the hST8Sia I promoter that was crucial for FenR-induced gene expression. We isolated the region between -1146 and -646 as the core promoter; this region was required for transcriptional activation of hST8Sia I in FenR-stimulated SH-SY5Y cells. In the previous studies, we have shown several transcription factor binding sites such as c-Ets-1, AP-1, CREB and NF-κB binding sites in this region [7-9]. We have also demonstrated that only the NF-κB binding site at position at -731 to -722 in this region contributes to hST8Sia I promoter activity in Fas-induced Jurkat T cells [7], human melanoma SK-MEL-2 cells [8], and valproic acid-induced human neuroblastoma SK-N-BE(2)-C cells [9]. In agreement with these findings, our present promoter assay with site-directed mutagenesis indicated that binding to this NF-κB element mediated FenR-dependent up-regulation of hST8Sia I gene expression.

NF-κB is a crucial transcription factor that controls the expression of various genes involved in immune and inflammatory responses, cell cycle progression, apoptosis, and oncogenesis [1,3]. Although it has been previously reported that the proapoptotic activity of FenR is mediated by NF-κB in SH-SY5Y cells [2], signaling pathway responsible for NF-κB activation by FenR stimulation in human neuroblastoma cells have not been reported. Therefore, it is important to elucidate which signaling pathways are upstream of this NF-κB-mediated enhanced expression of the hST8Sia I gene. In the present study, our data showed that AKT activation was induced by FenR in SH-SY5Y cells, suggesting that transcriptional activity of hST8Sia I may be related to AKT-dependent pathway induced by FenR in SH-SY5Y cells, which has not been reported yet. FenR stimulation enhanced the protein level of NF-κB p65 in nuclear fraction of SH-SY5Y cells. Taken together, these suggest that the activation of NF-κB and its translocation to nucleus in FenR-induced SH-SY5Y cells is readily reconciled with the hST8Sia I gene expression and increase of GD3 level in these cells.

Although the precise mechanisms involved in the constitutive activation of NF-κB leading to a transcriptional up-regulation of the hST8Sia I gene are unknown, we have demonstrated here for the first time that the AKT-dependent NF-κB activation regulates the expression of hST8Sia I in FenR-stimulated SH-SY5Y cells.

Acknowledgment

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초록 : 사람 신경모세포종 세포주 SH-SY5Y에서 fenretinide에 의한 GD3합성효소(hST8Sia I)의 전사조절기작

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(동아대학교 생명자원과학대학 생명공학과)

사람 신경모세포종 세포주 SH-SY5Y에서 Fenretinide (FenR)에 의한 GD3합성효소(hST8Sia I)의 발현증가기작을 규명하게 위하여 hST8Sia I의 프로모터 활성을 조사해 본 결과 -1146에서 -646영역에서 FenR에 의한 활성증가를 나타내었다. 또한 부위특이적 변이의 분석은 -731에서 -722영역에 위치한 전사인자 NF-kB 결합부위가 hST8Sia I의 FenR에 의한 활성증가에 중요하게 관여하고 있음을 나타내었다. FenR에 의한 hST8Sia I 유전자의 발현유도에 포함된 신호전달기작을 전사인자 단백질의 항체를 이용하여 조사해 본 결과 FenR처리에 의해 세포질에서는 인산화된 AKT단백질 수준의 증가가 관찰되었고 핵내에서는 NF-kB의 p65단백질의 증가가 관찰되었다. 이러한 결과들은 FenR에 의한 hST8Sia I 유전자의 발현증가는 AKT신호전달경로에 의해 활성화된 NF-kB의 핵내로 이동하여 hST8Sia I 유전자의 프로모터에 결합함으로서 전사가 촉진되어 일어난다는 것을 나타낸다.