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25-Hydroxy Vitamin D Exhibits NGF-like Activity in PC12 Cells

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Abstract

Background: In our previous study, 25-hydroxy vitamin D (25OHD) supplementation was associated with improved serum vitamin D levels and, possibly, improved cognitive function. In response to nerve growth factor, PC12 cells first undergo proliferation, followed by growth arrest and differentiation. We studied the effects of 25OHD on neurite elongation in PC12 cells.

Methods: PC12 cells were treated with 10ng/mL NGF with or without 6 nmol/L of 25OHD for 9 days. Phase-contrast micrographs were recorded and cells with a neurite outgrowth longer than the diameter of their cell body were counted. To investigate the expression level of Akt protein, PC12 cells were preincubated with 10ng/mL NGF with or without 6 nmol/L of 25OHD for 4 days, and lysates were assayed using a commercially available AKT/AKT ELISA kit.

Results: In the presence of NGF, 25OHD induced an increase in both the number of cells with extended neurites and the length of neurites. Expression of phosphorylated Akt was enhanced by 25OHD without NGF and 25OHD up-regulated NGF-induced phosphorylation.

Conclusion: These findings show that 25OHD displays NGF-mimicking and NGF-enhancing neurogenic activity in PC12 cells, which potentially depended on activating P3K/AKT signaling pathways.

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Introduction

Vitamin D is a liposoluble pleiotropic hormone that is mainly synthesized in the skin from cholesterol precursors upon exposure to solar 280-315nm (UVB) radiation, or acquired from dietary sources such as oily fish [1].

Vitamin D is a secosteroid associated with peripheral calcium homeostasis and nervous system function [2]. Vitamin D is available in two forms, vitamin D2 from plants and D3 from animals. Both vitamin D2 and D3 are biologically inert and require activation through two hydroxylation processes involving 25-hydrooxylase (CYP2R1) and 1α-hydroxylase (CYP27B1), located in the liver and kidney, respectively [3]. 1, 25-dihydroxyvitamin D is a biologically active metabolite produced by two hydroxylation reaction steps in the nervous system [4].

However, low 25-hydroxy vitamin D (25OHD) levels were recently associated with greater risk of cognitive impairment in older as well as younger adults using the Montreal Cognitive Assessment (MoCA) Arabic version [5].In a previous study, we showed that 25OHD supplementation was associated with improved serum vitamin D levels and possibly improved cognitive function [6].

Rat pheochromocytoma-derived cell line PC12 cells undergo proliferation followed by growth arrest and differentiation in response to nerve growth factor [7]. One aim of the present study was to show that 25OHD act directly on neuronal cells.

Akt is activated by a variety of mitogen factors and cytokines as well as NGF [8]. Serine / threonine kinase (Akt) is involved in the neuronal differentiating effect of nerve growth factor (NGF) [9]. Another aim of the present study was to show that the phosphatidylinositol 3-kinase/ AKT (PI3K/AKT) signaling pathway is involved in the 25OHD induced neurogenic activity.

Therefore, the present study was designed to investigate the effect of 25OHDonNGF induced neurite elongation and phosphorylated AKT (Ser473)/ total AKT in PC12 cells.

Materials and Methods

Products

NGFβ from rats was purchased from (Sigma, USA). 25OHD was purchased from Immundiagnostik AG (Bensheim, Germany).

Cell culture and differentiation

The rat adrenal pheochromocytoma-derived cell line PC12 was obtained from RIKEN Bio Resource Research Center (Ibaraki, Japan) and maintained in Dulbecco's modified Eagle's medium (Thermo Fishier Scientific, Japan), supplemented with 10% deactivated horse serum, 10% deactivated bovine serum, 100 unit/mL penicillin and 100 µg/mL streptomycin sulfate, at 5% CO_2, 37 °C. PC12 cells were seeded at a density of 1.5 x 10^5 cells. After 24h of incubation, the PC12 cells were treated with 10ng/mL NGF with or without 6 nmol/L of 25OHD for 9 days.

Measurement of neurite elongation

Phase-contrast micrographs were recorded on a personal computer using a charge coupled device camera (Ikegami, Japan), followed by analysis using software (Miotic Images Plus 2.1S; Shimadzu, Japan). Cells with protrusions longer than the cell body diameter were counted as cell with neurites. PC12 cells were treated with NGF for 9 days for the proper tracing of neurites belonging to a specific cell body.

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Phosphorylated AKT / total AKT (pAKT/AKT) assay

Cells were treated with 10ng/mL NGF with or without 6 nmol/L of 25OHD for 4 days and washed 3 times with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation buffer (RIPA) and protease inhibitor (Santa Cruz Biotechnology, USA). Lysates were centrifuged for 10 min at 12,000 g and supernatants were analyzed for protein concentration using Pierce™BCA Protein Assay kit (Peirce, Rockford, IL). pAKT/AKT were assayed using the commercially available ELISA kit (AKT1/2/3(pS473)+AKT1 Total SimpleStep ELISA™ Kit, Abcam, UK).

Statistical analyses

The data are expressed as mean ±SE, and the mean values of each group were compared in a one-way analysis of variance. A p-value of < 0.05 was considered to be statistically significant. Analyses were carried out using SPSS 21 for Windows (IBM, Japan).

Results

Effect of 25OHD on NGF-induced neurite formation of PC12 cells

PC12 cells are known to differentiate into sympathetic nerve-like cells and show neurite outgrowth in the presence of NGF [7]. The morphological changes in PC12 cells after treatment with 6 nmol of 25OHD and 10 ng/mL NGF are displayed in Figure 1A.

25OHD induced an increase in the length of neurites in the presence of NGF (Figure 1B). These results indicate that 25OHD exhibited NGF-enhancing activity in PC12 cells.

Effect of 25OHD on phosphorylation level of Akt

Akt is involved in the neuronal differentiating effect of NGF [10]. Akt is activated by a variety of mitogen factors and cytokines as well as NGF [8]. In this study, NGF enhanced phosphorylation of Akt (Figure 2). Expression of phosphorylated Akt was enhanced by 25OHD without NGF and 25OHD up-regulated NGF-induced phosphorylation (Figure 2).

PC12 cells were cultured with NGF for 4 days with and without 6 nmol/L of 25OHD. Lysate was subjected to the pAkt/Akt assay. Each experiment was repeated three times. Data are expressed as pAKT/AKT (%) and mean±SE.

Discussion

Many studies have shown that NGF enhances neurite outgrowth and Akt phosphorylation [7,8,10]. In our study, 25OHD was also shown to enhance NGF-induced acetylcholinesterase activity [11].

In this study, 25OHD enhanced NGF-induced neurite growth of PC12 cells and up-regulated NGF-induced Akt phosphorylation.

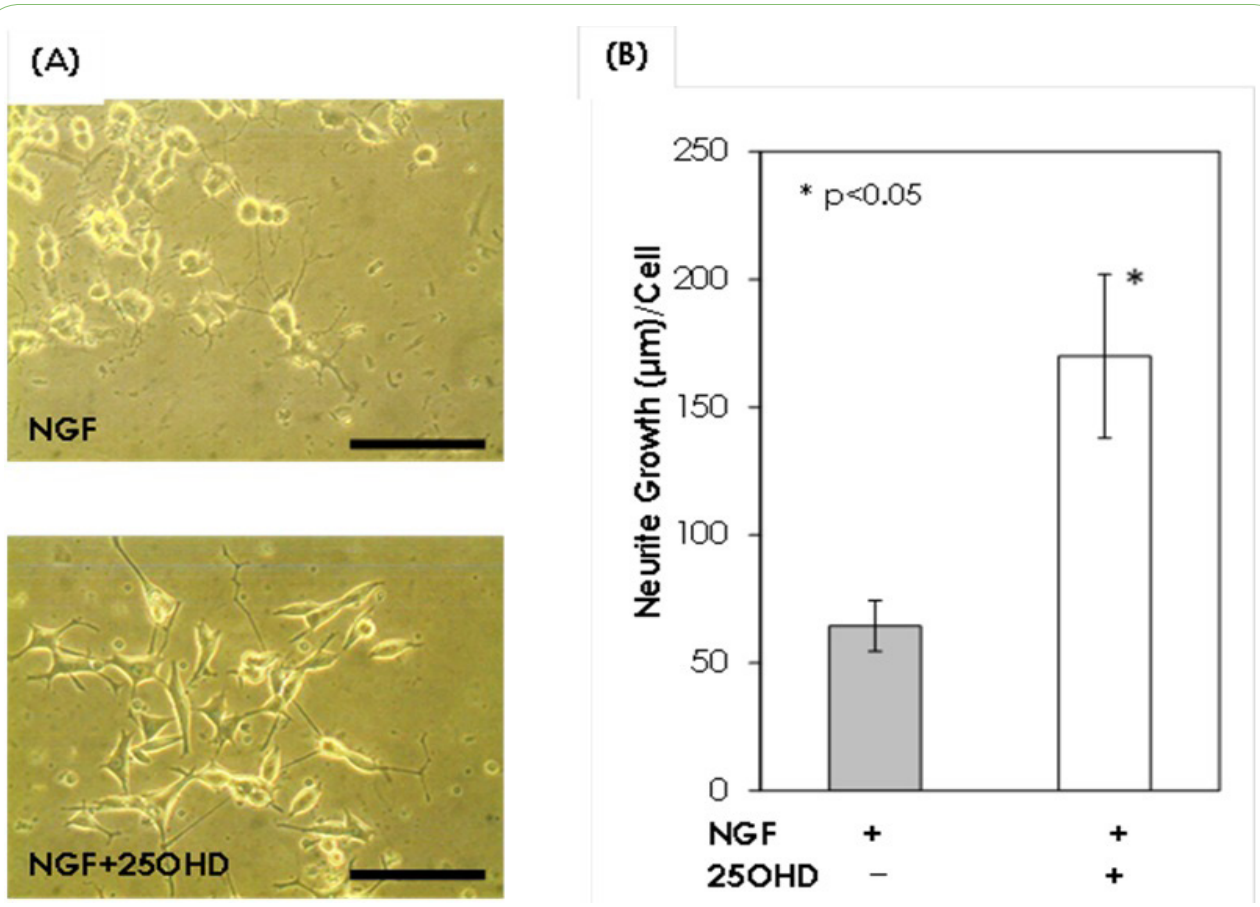

Figure1: Neurite growth in PC12 cells.
(A): Phase-contrast photomicrographs. PC12 cells were treated with 10 ng/L NGF in the medium for 4 days with and without 6nmol/L of 25OHD.
(B): Neurite outgrowth with and without 6 nmol/L of 25OHD. Each experiment was repeated three times. Scale bar= 100 μm.
Data are expressed as mean±SE. # indicates significant differences at $p< 0.05$.

These results suggest a potential role of 25OHD in activating protein kinase B (Akt), and in NGF-enhancing and NGF-mimicking neurogenic activity.

Osteocalcin (OC) prevents neuronal apoptosis in the hippocampus [12]. OC crosses the blood-brain barrier, binds to neurons of the brainstem, midbrain, and hippocampus, and enhances the synthesis of monoamine neurotransmitters [12]. OC directly acts to promote cell proliferation and differentiation, and affects the phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2 in the stimulated PC12 cells [13]. Further study is needed on whether the ERK1/2 signaling pathway is involved in the 25OHD induced neurogenic activity.

Low vitamin D has been associated with a risk of developing Alzheimer's disease [14,15]. In our previous study, dementia patients treated with 25OHD for 6 months improved their performance on a cognitive test (MMSE and MoCA-J score) [6]. These results suggest that 25OHD acts directly to alleviate the impairment of cells in Alzheimer's disease through the blood-brain barrier.

However, no clear evidence on low vitamin D levels as a risk factor for Alzheimer's disease exists, since interventional studies are few [16].

Further study of optimal 25OHD levels for maintaining physical and cognitive functions is needed.

Conclusion

These findings indicate that 25OHD might have NGF-like activity in PC12 cells.

Competing Interests

The authors declare that they have no competing interests.

Author's Contribution

Ms. Mochizuki was responsible for data acquisition and proof-reading of the manuscript and she participated in the data analysis.

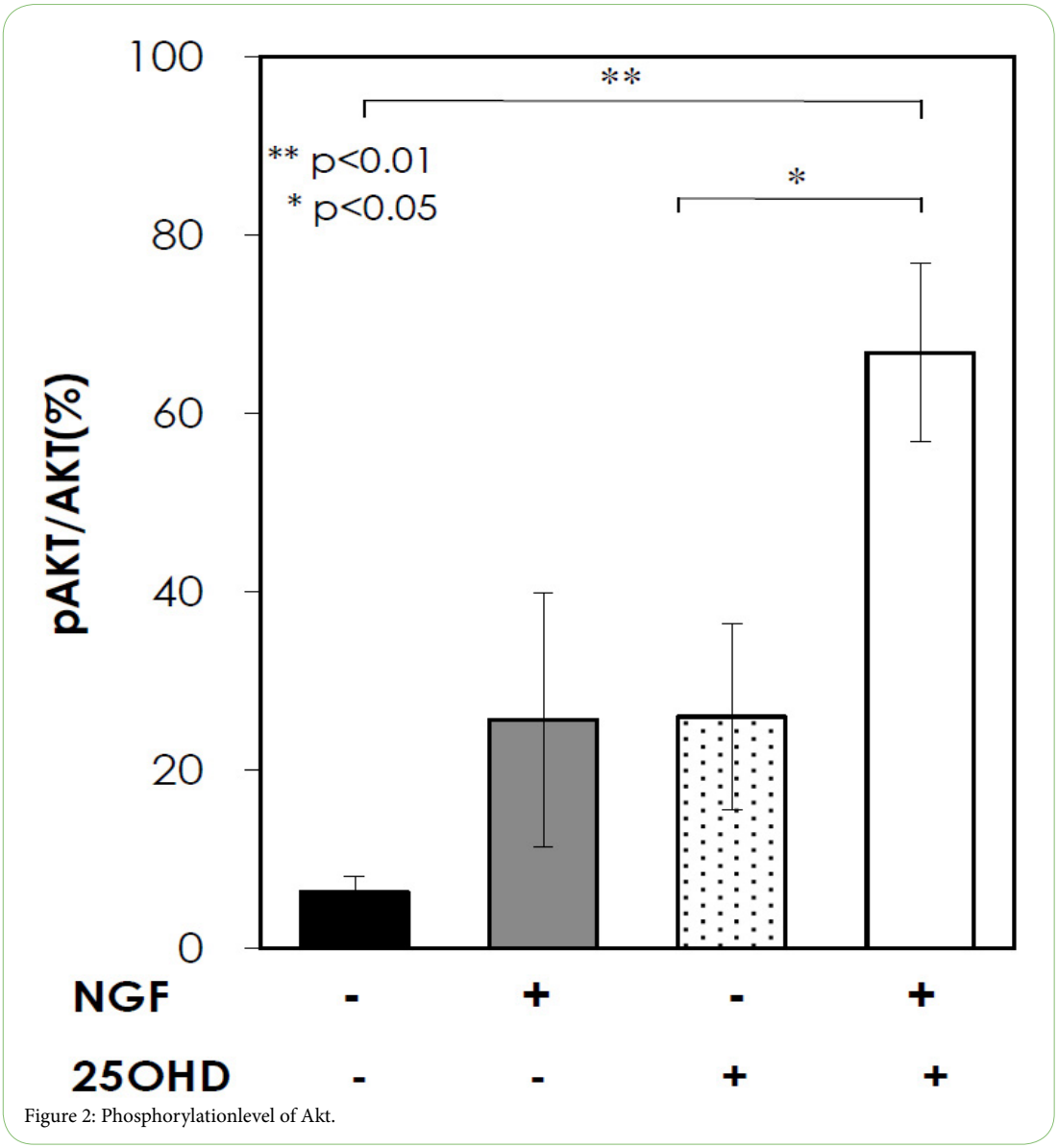

Figure 2: Phosphorylationlevel of Akt.

Dr. Hasegawa was responsible for the study conception, design, analysis, interpretation of data, and drafting of the manuscript.

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