

*Full Length Research Paper*

# Existence of endogenous ligand from secretory granules of bovine adrenal medulla for neurite outgrowth in PC12 cells

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Memory function is sensitive to the effects of aging, and memory loss is one of the most common complaints among the elderly population.. Proliferation and differentiation of cells in response to extracellular signals is influenced by the differential regulation of MAPKs. The PC12 cells are widely used as a cell system for the study of growth factor–stimulated cell functions, in which the intensity and duration of activation the ERK have been proposed to govern a distinct switch between cell proliferation and differentiation. In the present study we have purified a protein ligand from secretory granules of bovine adrenal medulla which significantly activates the phosphorylation of serine/threonine protein kinase (Akt) and Extracellular signal-Regulated protein Kinase (ERK) and stimulation of neurite outgrowth *in vitro*.

**Keyword:** Cells, Aging, Protein, Neurite outgrowth in vitro and Extracellular signals.

## INTRODUCTION

Many research reports have demonstrated that nerve growth factor (NGF) and other neurotrophic factors induce neurite outgrowth in sensitive types of cells and play a vital roles in the survival and growth of the neurons in the central nervous system (Greene and Tischler, 1976). Based on available research reports, neurotrophic factors are expected to have a regenerative effect in the injured tissues of neurodegenerative diseases such as Alzheimers disease and Parkinsonism. The exact mechanism for memory loss remains unknown because memory decline is either due to normal senescence of the brain or due to diseases that target memory regions of the brain. The hippocampal formation is important for long–term episodic memories of the brain and this region is targeted by diseases such as Alzheimer’s disease as well as by many physiological processes which change in an age-dependent manner.

PC12 cell line is a neuroendocrine cells derived from rat phaeochromocytoma which exhibits a wide variety of neuronal properties resembling those of sympathetic neurons in response to NGF (Greene et.al 1976). The PC12 cells are used as a good model system for studying

neuronal differentiation. PC12 cells extend neuron like processes and acquire characteristics similar to sympathetic neuron following treatment with NGF. Activation of serine/threonine kinase has been implicated in PI3-kinase mediated neuronal survival.(Kaplan 1992)

## UCSB Multidisciplinary Research on Magnetic Nanoparticle Guided therapy

The adrenal medulla, the central portion of the adrenal gland, contains a collection of sympathetic nerve cells that specialize in two important functions: (i) response to the sympathetic nervous system, particularly during stress and (ii) secretion of catecholamine hormones that are known to stimulate central nervous system in regulating metabolic and cardiac processes. In bovine adrenal medulla, secretory granule matrix contains a complex mixture of catecholamines, nucleotides, peptides and proteins. The major secretory proteins are chromogranin A, secretogranin I and II. Of the neuropeptide in adrenal chromaffin granules, enkephalins and neuropeptide Y are quantitatively more important (Goumon et al., 2000). Several studies have addressed: (i) their structure and biochemical properties; (ii) their expression in endocrine cells and neurons; (iii) their

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intracellular localization and (iv) their possible roles as a helper proteins in the packaging of peptides, hormones, or neuropeptides and they also serve as precursors to biologically active peptides (Fischer,2003 ; Strub *et al.*,1995; and Bauer *et al.*,2000).In bovine chromaffin granules, chromogranin A is a predominant secretory protein and is processed to an extent of 50 percent where as secretogranin I is processed up to 85 percent Chromatogranin and secretogranins constitute 80 percent of total water soluble proteins and they are proteolytically processed into small peptides within the secretory granules and these peptides are co-released with catecholamines upon cell stimulation under stress condition.

The peptides derived from these secretory proteins by proteolytic processing is a topic of growing interest, since biological activities have been attributed to specific domains located along their sequence. For example, Vasostatin-I and II, peptides derived from the N-terminal sequence of chromogranin A (1-76, 1-113) have been found to exhibit vaso-inhibitory activity in isolated human blood vessels (Aardal *et al.*,1993). Many research reports demonstrated that secretoneurin, a neuropeptide corresponding to secretogranin II (154-186), exhibits angiogenic properties *in vivo* and it also showed anti-apoptotic and proliferative effects on endothelial cells through the activation of Akt and MAPK pathway. Pancreastatin, a peptide derived from chromogranin A of pancreas counteracts insulin action in hepatocytes and adipocytes. A peptide corresponding to bovine chromogranin A, catestatin (344-364) inhibits catecholamine release from PC12 cells (Mehta *et al.*, 1993). Another chromogranin A derived peptide, located near the C-terminal domain of chromogranin A, parastatin (347-419), inhibits parathyroid cell secretion . Peptides derived from chromogranin-A such as chromofungin (47-66), vasostatin-I (1-76) and chromacin (173-194) are reported to have antibacterial activity against gram-negative bacteria and antifungal activity against yeast and filamentous fungi . Antibacterial activities are also reported from several peptides derived from Proenkephalin-A. (Goumon *et al.*, 2000). Hence, bovine adrenal chromaffin granule matrix is a potential source for the search of endogenous ligand for different biological activities.

## Materials and Methods

### Cell-culture

Rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Horse Serum (HS), 5% Fetal Calf Serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) under a humidified atmosphere containing

5% CO<sub>2</sub>.

### Preparation of Adrenal chromaffin granule lysate

Secretory granules were isolated from porcine adrenal medullae at 4° C according to Winkler and Fischer (1980) with slight modifications. Briefly, fresh bovine adrenal glands were collected from local slaughterhouse. Adrenal medullae were minced and homogenized with 0.3 M sucrose by using Polytron. Homogenates were centrifuged at 1000Xg for 10 min. and the supernatant was passed through cheesecloth. The supernatant was transferred to a new tube and centrifuged at 100,000Xg for 1 hr. Partially purified secretory granules formed a pellet in the base of the tube along with some mitochondrial debris. The crude granule pellet was resuspended in 0.3 M sucrose, layered on top of 1.6 M sucrose, and centrifuged at 100,000Xg for 2hr. Pink chromaffin granules were deposited at the bottom of the tube while mitochondrial impurities were separated at the interface between 0.3 M and 1.6 M sucrose gradient. The granule pellet was carefully collected and resuspended in 1mM Hebes buffer at pH 7. The granules were lysed by successive freezing and thawing. The lysed granules were centrifuged at 130,000Xg for 1 hr. The chromaffin granule membranes were deposited as pink pellet while the supernatant contain the granule lysate.

### Solid phase extraction

Soluble proteins/peptides from chromaffin granules were loaded onto 5 mL Hydrophilic Lipophilic Balance (HLB) cartridge, (Water oasis HLB extraction cartridge – 6cc/500mg) which was preconditioned in 1 mL Methanol and equilibrated with 1mL water. Cartridges were washed with 5% Acetonitrile (CH<sub>3</sub>CN)/0.1% Trifluoroacetic acid (TFA), and then eluted with 70% CH<sub>3</sub>CN/ 0.1%TFA.

### Reverse phase HPLC

Isolation of endogenous ligands was carried out on an AKTA explorer HPLC system (Amersham Pharmacia Biotech.) The concentrated eluent from the HLB cartridge was separated by using reverse phase Vydac Protein C4 analytical column. The solvent system consisted of 0.1% Trifluoroacetic acid in water (Solvent A) and 0.1% Trifluoroacetic acid in acetonitrile (Solvent B). Elution was performed at a flow rate of 0.5 to 0.75 mL/min. 1 mL fractions were collected. For western blot analysis 60µL fractions were concentrated by freeze drying and resuspended in 10µL of double distilled water. The active fraction 50 of C4 preparative column was loaded onto a C4 analytical reverse phase HPLC column. The solvent system consisted of 0.1% Trifluoroacetic acid in water

(Solvent A) and 0.1% Trifluoroacetic acid in acetonitrile (Solvent B). 500 $\mu$ L fractions were collected.

### Western blotting

To characterize phospho-ERK (Extracellular Regulated protein Kinase), PC12 cells treated with Nerve Growth Factor (NGF) or test peptide, 24 h after serum starvation the cells were detached from culture dishes by treatment with trypsin-EDTA, washed with serum-free DMEM containing 1% BSA, and resuspended in the same medium. Cells were allowed to spread on 100-mm tissue culture dishes coated with 10  $\mu$ g/ml of collagen I for 1 h. After 5 min of NGF or test peptide treatment, cells were washed in ice-cold (Phosphate Buffered Saline)PBS, and solubilized in the lysis buffer, and total lysates were then detected by the immunoblotting of antiphospho-ERK antibody respectively.

## RESULTS

### Effect of endogenous ligand on serine/threonine protein kinase (Akt) and Extracellular Signal-Regulated protein Kinase (ERK) activation

PC12 cells treated with 60  $\mu$ L of C4 preparative chromatographic fractions were assayed for the activation of Akt. As indicated in Figure 1 substantial increase in Akt activity was observed in following fractions; 49-51, 55,56 and 58-60. The active fraction 50 was further purified by using C4 analytical reverse phase column. PC12 cells exposed to 25  $\mu$ L of C4 analytical chromatographic filtration fractions were assayed for effects on Akt activity. As shown in Figure 2, treatment of chromatographic fractions resulted in marked increase in Akt activity. The active fractions were observed in 13-15, 17,20 and 21.

To show the effect of endogenous ligand on ERK activation, PC12 cells were treated with 60  $\mu$ L of C4 analytical HPLC fractions. As depicted in Figure 1, ERK activation was remarkably increased in several fractions which were grouped into three different ligands such as 49-51, 55,56 and 58-60. The active fraction 50 was further purified by using C4 analytical reverse phase column and PC12 cells exposed to 25  $\mu$ L of C4 analytical chromatographic filtration fractions were assayed for effects on ERK activity. As shown in Figure 2, treatment of chromatographic fractions resulted in marked increase in ERK activity in fractions such as 13-15, 17,20 and 21.

### Effect of endogenous ligand on neurite outgrowth

The active fraction 15 of reverse phase C4 analytical

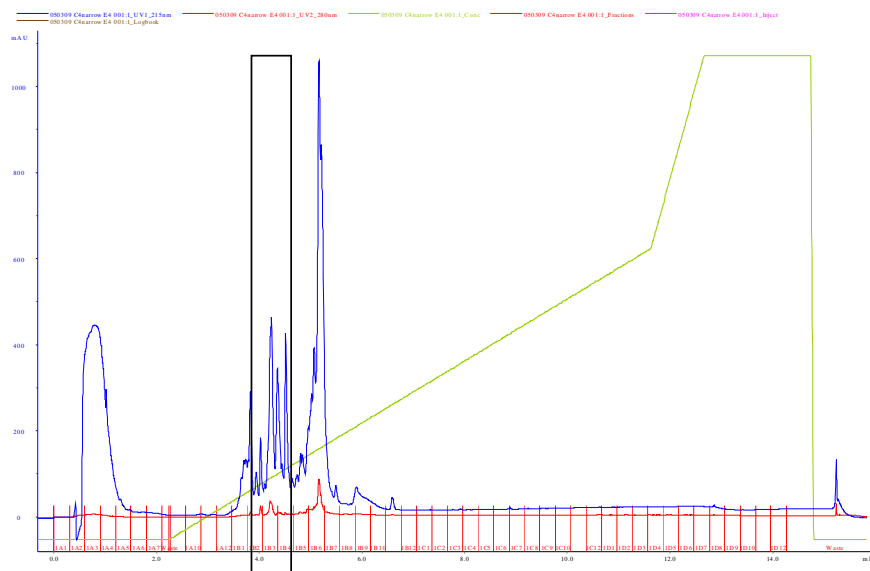
HPLC fraction was tested for the stimulation of neurite outgrowth in PC12 cells with Pituitary Adenylate Cyclase Activating Peptide as a positive control. The endogenous ligand was significantly activating the neurite outgrowth.

## DISCUSSION

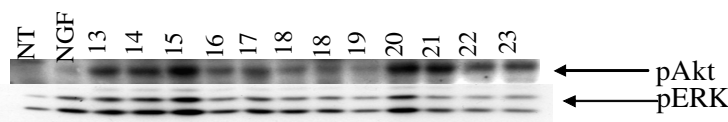
Secretory granules of bovine adrenal medulla contain chromogranin A, secretogranin I and II and proenkephalin A as major high molecular mass proteins. Chromogranin A (48 KDa), Secretogranin I (71KDa) and II (64 KDa) and proenkephalin A (29 KDa) are considered as precursor proteins that are proteolytically processed into small peptides. Different biological activities in relation to the autocrine or paracrine role in hormone secretion and antimicrobial activities have been attributed to peptides located along the sequence of these secretory proteins are reported (Strub et al.,1995 and Bauer et al.,2000). Since these peptides are co-released with catecholamines under stressful situations, it was thought that these peptides may have important role in defense mechanism. Several peptides such as vasostatin-I, chromofungin, chromacin (chromogranin A), secretolytin (secretogranin-I) and enkalyptin (proenkephalin-A) are reported to have antibacterial activity against gram positive bacteria and antifungal activity against filamentous fungi and yeast. Besides antimicrobial activities, vasostatin-I exhibits other biological activities such as inhibition of parathyroid hormone secretion, regulation of cell adhesion and the neurotoxicity in neuronal/microglial cell co-cultures.

Western blot data showed that ERK activation was significantly enhanced in HN33 cells treated with ligands separated by reverse phase C4 analytical chromatography and gel filtration chromatography. Many research reports suggested that ERK plays an important role to regulate diverse neuronal process such as cell proliferation, differentiation, synaptic plasticity and cell death. Berman *et al* (1980) described that inhibition of ERK activation by microinjection of MEK inhibitor into the insular cortex of rat resulted in the attenuation of long-term memory. In 1998 Atkin et al, found that ERK activation was required for the learning behavior of rodents.

In the present study, we have observed the activation of Akt and ERK phosphorylation by several reverse phase chromatographic fractions of secretory granule lysate from bovine adrenal medulla. In this activation scenario as observed from the peak fraction of the HPLC it is deduced that the analytical reverse phase chromatographic fraction 15 is significantly stimulating the neurite growth in PC12 cells *in vitro*. The identification of the active ligand was proposed as peripheral benzodiazepine ligands which influence ornithine decarboxylase (James Morgan et al 1985) This active ligand could serve as potential therapeutic drug for



**Figure 1**



**Figure 2.** Reverse phase analytical HPLC chromatographic fractions showing the phosphorylation of Akt and ERK in western blot

the control of neurodegenerative diseases such as Alzheimers disease and Parkinsonism. The relevance of ERK activation in PC12 by the secretory ligand to neurite outgrowth was prominent. The activation of ERK receptors coupled to phosphoinositide hydrolysis releases cytosolic inositol 1,4,5-trisphosphate (InsP3), which discharges calcium from intracellular stores. The subsequent depletion of intracellular calcium stores by InsP3, or alternatively loss of stores by inhibition of sarcoplasmic reticulum/endoplasmic reticulum (SERCA) type-Ca<sup>2+</sup>-ATPases by selective inhibitors

### CONCLUSIONS AND SUGGESTIONS

Memory function is sensitive to the effects of aging, and memory loss is one of the most common complaints among the elderly population. Proliferation and differentiation of cells in response to extracellular signals is influenced by the differential regulation of MAPKs. The PC12 cells are widely used as a cell system for the study of growth factor-stimulated cell functions, in which the

intensity and duration of activation the ERK have been proposed to govern a distinct switch between cell proliferation and differentiation. The analytical reverse phase chromatographic fraction 15 is significantly stimulating the neurite growth in PC12 cells *in vitro*. The identification of the active ligand was proposed as 1 benzodiaaphene ligands which influence ornithine decarboxylase. This active ligand could serve as potential therapeutic drug for the control of neurodegenerative diseases such as Alzheimers disease and Parkinsonism. In the present study we have purified a protein ligand from secretory granules of bovine adrenal medulla which significantly activates the phosphorylation of serine/threonine protein kinase (Akt) and Extracellular signal-Regulated protein Kinase (ERK) and stimulation of neurite outgrowth *in vitro*. The analytical reverse phase chromatographic fraction 15 is significantly stimulating the neurite growth in PC12 cells *in vitro* was observed from the peaks in the chromatograph which made the fraction 15 dominant and active in presence of other ligands

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