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# Phospholipase D is Dispensable for Epidermal Growth Factor-Induced Chemotaxis

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**α-Synuclein (α-Syn) is implicated in several neurodegenerative disorders, including Parkinson's disease, known collectively as the synucleinopathies. α-Syn is known to be secreted from the cells and may contribute to the progression of the disease. Although extracellular α-Syn is shown to impair platelet-derived growth factor-induced chemotaxis, molecular mechanism of α-Syn-induced motility failure remains elusive. Here we have aimed at phospholipase D (PLD) as a potential target for α-Syn and examined the involvement of this enzyme in α-Syn action. Indeed, extracellular α-Syn caused inhibition of agonist-induced PLD activation. However, inhibition of hydrolytic activity of PLD by 1-butanol treatment showed little or no effect on agonist-induced chemotaxis. These results suggest that some signaling pathways other than PLD may be involved in α-Syn-induced inhibition of chemotaxis.**

## INTRODUCTION

Cell migration is vital for regulation of cell and tissue homeostasis. It is particularly important in physiological processes such as embryogenesis, wound healing, and neuronal functions. The migration process involves signaling cascades starting from a variety of growth factors and cytokines, their receptor activation, membrane lipid remodeling, and activation of various enzymes leading to fine tune of orchestration of actin filament remodeling necessary for cell movement. However, the signaling pathways responsible for coordinating these processes are not fully understood.

Lipid metabolites such as phosphatidic acid (PA) have been shown to play an important role in chemotaxis in a variety of cell types (1). PA is mainly produced by a phosphatidylcholine-hydrolyzing enzyme, phospholipase D (PLD). In mammals, two PLD isozymes, PLD1 and PLD2, have been identified and have ~50% sequence homology (2). PLD can be activated by various signaling molecules such as lipids, *e.g.*, phosphatidylinositol 4,5-bisphosphate (3) and phosphatidylethanolamine (4) and by proteins, *e.g.*, ADP-ribosylation factor (3), $G_{M2}$ activator (5, 6) and CtBP1/BARS (7) and inhibited by α-Synuclein (α-Syn) (8).

α-Syn, a 140-amino acid protein of undefined function, is implicated in several neurodegenerative disorders, including Parkinson's disease (PD), known collectively as the synucleinopathies (9). Recent lines of evidence suggest that cell-to-cell propagation of α-Syn plays a role in the progression of PD symptoms from initially peripheral nervous system and olfactory bulb affected, ascending toward the brainstem and the midbrain such as substantia nigra, and then eventually spreads to the forebrain as suggested by "Braak's hypothesis" (10). It remains elusive as to how extracellular α-Syn participates in the pathogenesis of PD.

Recently we have found that extracellular addition of α-Syn to human neuroblastoma SH-SY5Y cells causes suppression of platelet-derived growth factor (PDGF)-induced chemotaxis with the potency being stronger in a mutant α-Syn(A53T) found in familiar PD than in the wild type. Furthermore, this α-Syn(A53T)-induced chemotaxis failure was closely associated with the inhibition of Rac1 activation, whereas Cdc42 activation remained unaffected, resulting in unbalanced actin filament remodeling (11). However, mechanism underlying extracellular α-Syn(A53T)-induced chemotaxis failure remains unclear.

It has been shown that PLD-catalyzed formation of phosphatidic acid is important in the translocation of Rac1 to plasma membranes for cell migration (12). From these backgrounds we have reasoned that extracellular α-Syn(A53T) may inhibit PLD, which results in the inhibition of Rac1 activation leading to chemotaxis failure. In the present studies we have undertaken experiments on the comparison of the effects of PLD inhibition and α-Syn(A53T) treatment on growth factor-induced chemotaxis. Unexpectedly, we found that α-Syn(A53T) affects as yet unidentified pathway(s) rather than PLD inhibition for chemotaxis failure.

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## MATERIALS AND METHODS

### Materials

Epidermal growth factor (EGF) was purchased from Sigma Aldrich; Insulin and nerve growth factor (NGF) from Wako; 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) from Santa Cruz Biotechnology. Other reagents and chemicals were of analytical grade.

### Bacterial expression and purification of recombinant α-Syn(A53T)

Recombinant α-Syn(A53T) was expressed in E. Coli and purified as described previously (13).

### Cell cultures and transfections

SH-SY5Y and COS7 cells obtained from American Type Culture Collection (ATCC, CRL-2266) and Riken Cell Bank (RCB0539) were maintained in DMEM/F-12 medium (Wako Pure Chemical Industries) and DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO2, respectively.

### Cell migration

Cell migration was assessed as described previously (11). Briefly, SH-SY5Y or COS7 cells in serum-free DMEM/F-12 or DMEM containing 0.1% fatty acid-free bovine serum albumin, respectively, were seeded on the collagen-coated upper wells of 24-well Transwell plate (Corning) in the absence or presence of 1 μM α-Syn(A53T), 0.3% 1-butanol, or 1μM FIPI. After 18 hr at 37 °C the cells migrated into the lower wells, where 100 ng/ml insulin, 100 ng/ml NGF, 100 ng/ml EGF or 20 ng/ml PDGF was present as specified in the Figure legends, were detached from the membranes and counted for the cell number by a hemocytometer.

### Membrane ruffling assay

COS7 cells were transfected with GFP-β-actin and serum-starved for 18 hr in the absence or presence of 1 μM α-Syn(A53T). In some experiments cells were pretreated with 0.3% 1-butanol for 10 min. Cells were then treated with or without 100 ng/ml EGF for 8 min in the absence or presence of 0.3% 1-butanol or 1 μM α-Syn(A53T), followed by fixation. The cells were analyzed using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

### PLD assay

COS7 cells were metabolically labeled with [$^{14}$C]lysophosphatidylcholine (0.5 mCi/1 x $10^7$ cells; PerkinElmer) for 18 hr in the absence or presence of 1 μM α-Syn(A53T) in serum-free DMEM. After washing with phosphate-buffered saline, PLD reaction was initiated by adding DMEM containing 0.3% 1-butanol in the absence or presence of 100 ng/ml EGF at 37 °C for 10 min. Termination of the reactions, lipid extraction and lipid separation were carried out as described earlier (14). PLD activity was expressed as a percentage of [$^{14}$C]PtdBut in the total radioactivity found in all spots in one lane.

## RESULTS

The effect of extracellular α-Syn(A53T) on various growth factor-induced chemotaxis of human neuroblastoma SH-SY5Y cells was studied using a two-chamber method. Insulin-induced chemotaxis was inhibited by α-Syn(A53T) by 30% (Figure 1A). NGF-induced chemotaxis was strongly (88%) inhibited by α-Syn(A53T). Similarly, EGF-induced chemotaxis was suppressed (40%) by this Parkinson's disease relevant protein. Recently, studies from our laboratory have revealed that extracellular α-Syn(A53T) causes inhibition of PDGF-induced chemotaxis by 30% (11). These results indicate that extracellular α-Syn(A53T) inhibits chemotaxis induced by a variety of growth factors although the extent of inhibition varies from growth factors and that it may inhibit a common signaling pathway leading to chemotaxis. In addition, this phenomenon was observed not only in neuronal cells such as SH-SY5Y (11) and PC12 cells (data not shown) but in non-neuronal cells such as COS7 cells (Figure 1B).

To dissect signaling pathways which are both α-Syn(A53T)-sensitive and cell motility-directing, one of the versatile signaling enzymes, PLD, was studied in relevance to extracellular α-Syn(A53T) action. Mammalian PLD is known to possess two distinct enzymatic reactions, *i.e*. hydrolytic and transphosphatidylation reactions producing phosphatidic acid and phosphatidylalcohol, respectively. PLD activity was assessed by the formation of phosphatidylbutanol in the presence of a primary alcohol, 1-butanol. In agreement with a previous report (8), EGF-stimulated PLD activation was inhibited by α-Syn(A53T) (Figure 2).

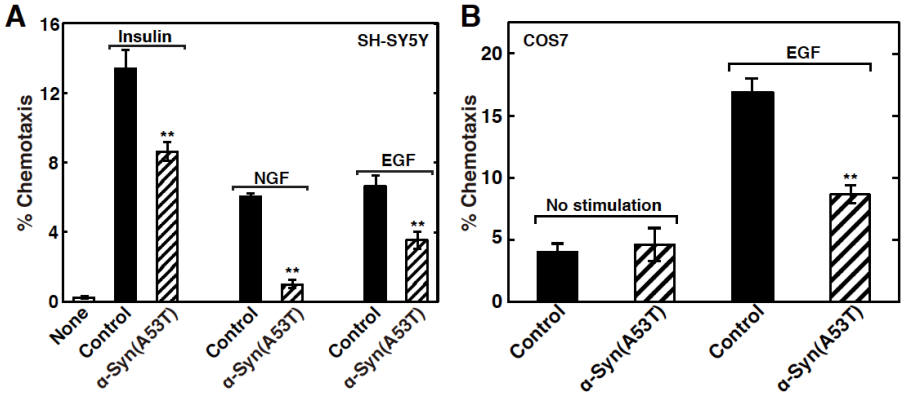


**Figure 1. Effect of extracellular α-Syn(A53T) on agonist-stimulated chemotaxis.**
SH-SY5Y (A) and COS7 cells (B) were cultured without serum for 18 hr in the upper chamber without (buffer control) or with 1 μM α-Syn(A53T). Cells which migrated into the lower chamber in the absence (none) or presence of 100 ng/ml insulin, 100 ng/ml NGF, or 100 ng/ml EGF were counted. Data are means ± s.e.m. of three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's t-test (n=9, **P<0.01 versus control (closed)).

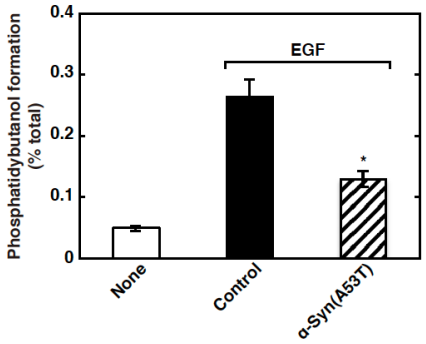


**Figure 2. Effect of extracellular α-Syn(A53T) on EGF-induced PLD activation.**
COS7 cells were metabolically labeled with [$^{14}$C]lysophosphatidylcholine for 18 hr and assayed for EGF-stimulated PLD activity in the presence of 0.3% 1-butanol without or with 1 μM α-Syn(A53T). PLD activity was expressed as percentage of the radioactive [$^{14}$C]PtdBut in the total radioactivity found in all spots in the lane obtained from autoradiography. Data are means ± s.e.m. of three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's t-test (*P<0.05 versus control (closed)).

To date most of the physiological actions of PLD are believed to be mediated by PLD-catalyzed formation of phosphatidic acid. The next experiments were conducted to evaluate the importance of phosphatidic acid in EGF-induced chemotaxis. PLD-catalyzed formation of phosphatidic acid was suppressed by adding 1-butanol to promote transphosphatidylation reactions at the expense of phosphatidic acid formation. Importantly, 1-butanol had no obvious changes in EGF-induced chemotaxis in contrast to α-Syn(A53T) treatment (Figure 3, compare with Figure 1). Moreover, PLD-specific inhibitor FIPI had no effect on EGF-induced chemotaxis. These results indicate that EGF-stimulated PLD activation is not essential to the downstream pathway leading to chemotaxis, which is consistent with a previous report (15).

It has recently been demonstrated that extracellular α-Syn(A53T)-induced chemotaxis failure is mainly ascribed to selective impairment of one of Rho family GTPases, Rac1 activation (11). Since it is well known that Rac1 is mainly involved in membrane-ruffle formation (16), the role of phosphatidic acid in EFG-induced membrane ruffling was assessed in comparison with α-Syn(A53T) effect. EGF induced a prominent membrane ruffling (Figure 4A), which was strongly inhibited by α-Syn(A53T) treatment. 1-Butanol treatment showed no obvious changes, which was clearly shown in quantitative results (Figure 4B). Taken together, these results indicate that EGF-stimulated formation of phosphatidic acid plays a dispensable role in extracellular α-Syn(A53T)-induced chemotaxis failure.

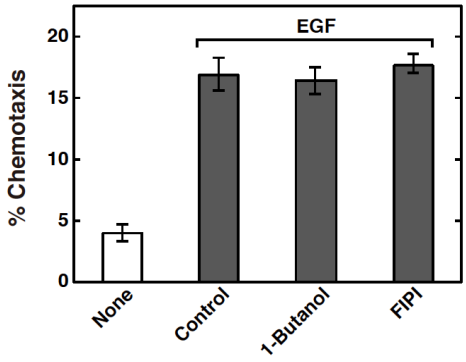


**Figure 3. Effect of 1-butanol on EGF-stimulated chemotaxis.**
COS7 cells were cultured for 18 hr in serum-free medium with 0.3% 1-butanol or 1 μM FIPI in the upper chamber and the cells migrated into the lower chamber in the absence (none) or presence of 100 ng/ml EGF were counted. Data are means ± s.e.m. of three independent experiments carried out in triplicate.

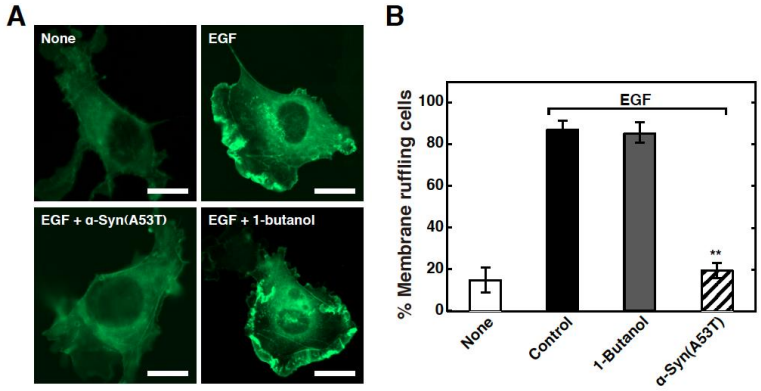


**Figure 4. Effect of 1-butanol on EGF-induced membrane ruffling.**
COS7 cells transfected with GFP-β-actin, which had been cultured in the absence of serum for 18 hr without or with 1 μM α-Syn(A53T), were stimulated for 8 min with buffer vehicle (none) or with 100 ng/ml EGF in the absence or presence of 0.3% 1-butanol. After fixation, the cells were analyzed using a confocal laser scanning microscope. The results are the representative of 3 independent experiments (A). Bars, 10 μm. Ruffling positive cells were quantitated and expressed as % membrane-ruffling cells per total (n=100) (B). The cells were counted ruffling-positive when more than 30% of total membrane areas were ruffled. Statistical significance was analyzed by Student's t-test (**P<0.01 versus control (closed)).

## DISCUSSION

α-Syn is enriched in synaptic terminals and is implicated in synaptic function. Further studies have revealed that α-Syn can be released from cultured cells by exocytosis (17) or by exosomes (18) and that α-Syn is detected in cerebrospinal fluid and plasma (19, 20). It is known that recombinant α-Syn is internalized by cultured neuronal cells, either by direct diffusion across the plasma membranes or through an endocytic pathway (21) and that Lewy body-like pathology propagates from host to grafted neurons in subjects with PD patient (22, 23). From this background, we reasoned that extracellular effect of α-Syn will give us some potential hints for the understanding of pathogenesis of Parkinson's disease.

We have shown in the present studies that extracellular α-Syn(A53T) causes suppression of chemotaxis induced by a variety of growth factors (Figure1) not only in neuronal (SH-SY5Y) cells (Figure 1A and a previous report (11)) but in non-neuronal COS7 cells (Figures 1B, 3, 4), suggesting that this may link to a common pathway to agonist-mediated cell motility. It has previously been shown that EGF receptor utilizes two downstream signaling, *i.e.*, Src family tyrosine kinase and phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3 kinase) cooperatively to promote maximal Rac1 activation for cell migration (24). Our previous study has shown that PDGF-induced PI3 kinase was not inhibited by extracellular α-Syn(A53T) in SH-SY5Y cells as

judged by Akt phosphorylation (11). Another important downstream signaling, PLD-catalyzed phosphatidic acid has been studied in the present study. Although EGF caused PLD activation and its activation was inhibited by extracellular α-Syn(A53T) (Figure 2), direct inhibition of phosphatidic acid formation by 1-butanol or by PLD inhibitor FIPI was not able to mimic α-Syn(A53T) action (Figure 3) at least in our conditions. These results may imply that EGF-induced PLD activation is dispensable with chemotaxis, although it may play a role in EGF-induced mitogenesis (24). Although we didn't assess cell proliferation in this study, it might be possible that extracellular α-Syn causes neurotoxicity through inhibition of PLD after long incubation. It has also been known that many growth factors utilize transactivation of S1P receptors for cell migration (25). Further studies in terms of S1P receptor transactivation as well as Src family tyrosine kinases are necessary to elucidate molecular action of extracellular α-Syn(A53T)-induced chemotaxis failure.

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