Identification of the amino acid sequence motif of α-synuclein responsible for macrophage activation

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ABSTRACT

Identification of the amino acid sequence motif of α-synuclein responsible for macrophage activation

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(Directed by Professor Jongsun Kim)

Chronic inflammation is associated with a broad spectrum of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (PD). Activated microglia and macrophages produce various inflammatory molecules that are associated with neuronal cell death. α-Synuclein (Syn) is implicated in the pathogenesis of PD and related neurodegenerative disorders. Recent studies have also shown that α-synuclein can activate microglia and enhance dopaminergic neurodegeneration. The mechanisms of microglia activation by α-synuclein, however, are not well understood.

In this study, we found that not only α-synuclein but also β- and γ-synucleins activated macrophages (RAW 264.7) *in vitro*. Macrophages treated with synuclein proteins secreted TNF-α and PGE_2 in a dose-dependent manner. Synuclein family proteins also increased mRNA transcription of COX2 and iNOS. Two α-synuclein deletion mutants, Syn ΔNAC and Syn 61-140, activated primary macrophages, as well as RAW264.7 cells, although the effect of syn 61-140 is less prominent than those of wild type and ΔNAC. The other deletion mutants Syn 1-60 and Syn 96-140 did not significantly activate macrophages. In addition, the macrophage activating effects of α-synuclein point mutants A53T, A30P, and E46K were slightly higher than that of wild-type α-synuclein. Finally, we demonstrated that macrophage activation by α-synuclein was accompanied by phosphorylation of ERK. These results suggest that synuclein family proteins can activate macrophages via the ERK phosphorylation, and that macrophage activation needs both the N-terminal and C-terminal domains of α-synuclein.

Key words : synuclein, macrophage, inflammation, TNF-α, PGE_2, Parkinson's disease

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I. INTRODUCTION

α-synuclein, an acidic, heat-resistant and unstructured protein of 140 amino acids long, is highly expressed in brain tissues and primarily localized at the presynaptic terminals of neurons [1, 2]. α-synuclein is widely expressed in the central nervous system (CNS), particularly the neocortex, hippocampus, striatum, thalamus and cerebellum. In addition, α-synuclein is expressed in various tissues, such as the

pancreas, kidney, skeletal muscles, lung, placenta, heart and blood cells, although it is less abundant than in the brain [2]. α-synuclein consists of three distinct regions. The N-terminal region (1-60 amino acid residues) contains KTKEGV repeats, which form amphipathic α-helices similar to the lipid binding domain of apolipoproteins. The central region (61-95 amino acids residues) is a very hydrophobic NAC (non Aβ-component of Alzheimer's disease) peptide, and the C-terminal region (96-140 amino acid residues) is primarily composed of acidic amino acids [2, 3]. In addition to α-synuclein, β-, γ- synucleins and synoretin, which belong to the synuclein family, have been identified in humans [4-7].

α-Synuclein has been identified as a major component of intracellular fibrillar protein deposits (Lewy bodies or glial inclusion bodies) in several neurodegenerative diseases, including Parkinson's disease [8, 9], diffuse Lewy body disease [10] and multiple systemic atrophy [11]. Interest in the pathological role of α-synuclein grew when several mutations (A30P, A53T and E46K) were found in some early onset familial Parkinson's disease patients [12-14], and animal models with transgenic overexpression of α-synuclein were shown to mimic several aspects of Parkinson's disease such as

neuronal loss and α-synuclein aggregation [15, 16]. Although the physiological role of α-synuclein remains elusive, gathering evidence indicates that α-synuclein is a key player in Parkinson's disease (PD) [17].

α-Synuclein has traditionally been considered a cytoplasmic protein [1]. This view was challenged recently by the finding that α-synuclein has been detected in extracellular biological fluids, including human cerebrospinal fluid (CSF) and blood plasma in both healthy subjects and patients with Parkinson's disease [18-20]. Recent studies have also shown that γ-synuclein, as well as α-synuclein, is present in CSF. In particular, α- and γ-synucleins are more abundant in aged subjects with neurodegenerative and vascular changes [21]. Moreover, a recent report demonstrated that α-synuclein is rapidly secreted from cells via unconventional, ER/Golgi-independent exocytosis [22], suggesting that extracellular α-synuclein may function physiologically or pathologically. In agreement with these suggestions, it has been recently reported that extracellular α-synuclein activates microglia, THP-1 cells and astrocytes [23-26]. Extracellular α-synuclein also induces microglial phagocytosis [27].

In the present study, we investigated the molecular mechanisms by which α-synuclein activates macrophages. To identify the amino acid sequence motif of α-synuclein responsible for macrophage activation, we used synuclein family proteins and several types of α-synuclein deletion mutants. We compared their influence on tumor necrosis factor-alpha (TNF-α) and prostaglandin E_2 (PGE_2) secretion, and looked at the expression of cytochrome c oxidase subunit 2 (COX2) and inducible nitric oxide synthase (iNOS) as macrophage activation markers. We also investigated the downstream signaling of α-synuclein-induced macrophage activation.

II. MATERIALS AND METHODS

1. Materials

Fetal bovine serum (FBS), RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM) were obtained from Gibco-Invitrogen (Carlsbad, CA, U.S.A.). Lipopolysaccharide (LPS), Thiazolyl blue tetrazolium bromide (MTT), Ampicillin, dimethyl sulfoxide (DMSO) and Polymyxin B sulfates were purchased from Sigma (St. Louis, MO, U.S.A.). ECL solution and BCA protein assay kits were purchased from PIERCE (Rockford, IL, U.S.A.). Antibodies to P44/42 mitogen activated protein kinase (MAPK), phospho-P44/42 MAPK, Stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), phospho-SAPK/JNK, p38 MAPK and phospho-p38 MAPK were obtained from Cell Signalling Technology (Danvers, MA, U.S.A.). Antibody against α-tubulin was obtained from Sigma (St. Louis, MO, U.S.A.). The ELISA TNF kit was from BD Biosciences (555212 and 558874, Franklin Lakes, NJ, U.S.A.) and the ELISA PGE_2 kit was from R&D systems (KGE004, Minneapolis, MN, U.S.A.). All other reagents used in this study were analytical grade and obtained from either Sigma (St. Louis, MO, U.S.A.) or USB (Cleveland, OH, U.S.A.).

2. Protein expression and purification

Synuclein proteins were overexpressed in *E. coli* (BL21), and the recombinant proteins were purified as described previously [28]. Briefly, the transformed bacteria were grown in LB medium with 0.1 mg/ml ampicillin at 37°C to an A600 of 0.8, and then cultured for an additional 4 h after being induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 8,000x*g* for 10 min, re-suspended in 20 mM MES pH 6.0, and then disrupted by ultrasonication. The supernatant was purified with DEAE anion-exchange chromatography, followed by CM cation-exchange chromatography in 20 mM MES, pH 6.0. The bound proteins were eluted with a linear gradient between 0.1 M and 0.5 M NaCl at a flow rate of 1.5 ml/min. All proteins were further purified on an FPLC gel-filtration column (GE healthcare, Uppsala, Sweden) pre-equilibrated with PBS, pH 7.4. All proteins were concentrated and buffer changed with a Centricon apparatus (Satorius Stedim Biotech, Goettingen, Germany). Proteins were quantitated with the BCA assay, filtered and stored at 4°C until use.

3. RAW 264.7 cell culture

RAW 264.7, a mouse macrophage cell line obtained from the Korean Type Culture Collection, was grown in DMEM with 10% FBS and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

4. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (1×10^5 per well) were cultured in 24 well plates overnight. The medium was then removed and replaced with fresh DMEM containing 10% FBS. Cells were incubated with the indicated doses of LPS or indicated doses of recombinant protein and 10 μg/ml polymyxin B sulfate for 18 h. The cell free supernatants were then harvested, and the TNF-α and PGE_2 levels in the supernatants were determined using ELISA kits according to each manufacturer's instructions.

5. Activation of primary human macrophages

Peripheral blood mononuclear cells (PBMC) from healthy volunteers were obtained through passage over Ficoll-hypaque gradient (GE Healthcare, Uppsala,

Sweden). The cells were washed and resuspended in RPMI 1640 medium with 10% FBS at a concentration of 4×10^6 cells/ml. These cells were placed on 24 well plates, at 1ml/well, and incubated for 2 h at 37°C in 5% CO_2. Nonadherent cells were removed from the plates, and adherent cells were cultured in RPMI 1640 medium with 10% FBS for 4 days. Cells were incubated with the indicated doses of LPS or recombinant synuclein proteins. 10 μg/ml of polymyxin B sulfate was added for 24 h. Then the cell free supernatants were harvested, and the TNF-α level in the supernatants were determined using ELISA kits according to each manufacturer's instructions.

6. MTT cell viability assay

RAW 264.7 cells (1×10^4 per well) cultured into 96-well plates were treated with saline (control), LPS alone (10 ng/ml), LPS with polymyxin B sulfates (10 μg/ml) or α-synuclein (0.1, 1, 5 and 10 μM) with polymyxin B sulfates for 18 h. After the incubation, MTT reagent (1 mg/ml per well) was added to the RAW 264.7 cell culture medium and incubated for 4 h. The medium was removed. DMSO (100 μl/well) was

added to each well and the plate was read at 570/630 nm in a microtiter plate reader (THERMO max, Molecular Devices, Melano-Park, CA, U.S.A.).

7. Reverse transcription polymerase chain reaction (RT-PCR)

RAW 264.7 cells (2×10^6) were incubated with the indicated doses of proteins for 2 or 6 h and harvested. Total RNA was isolated using an RNeasy mini kit (Qiagen, Santa Clara, CA, U.S.A.). The integrity of isolated total RNA was confirmed by 1.5% agarose gel electrophoresis. To synthesize cDNA, 1 μg of each RNA sample was mixed with 100 ng of random hexamers, 6 μl of 5x first strand buffer, 12 μl of 2.5 mM dNTPs (TaKaRa, Shiga, Japan) and 200 units of murine Molony leukemia virus reverse transcriptase (MMLV-RT) (Invitrogen, Carlsbad, CA, U.S.A.) and incubated at 42°C for 80 min. The reaction mixture was boiled at 95°C for 5 min, quickly chilled on ice, then used for PCR without further manipulation. cDNA was amplified by PCR using PCR PreMix (Bioneer, Seoul, Korea) and a pair of primers specific for the genes of interest. PCR was performed using the following specific oligonucleotide primer sets: COX-2 forward, 5′-TTC TTC AAC CTC TCC TAC TAC-3′, and reverse,

5′-GCA CGT AGT CTT CGA TCA CTA-3′; iNOS forward, 5′-ATG TCC GAA GCA AAC ATC ACA-3′, and reverse, 5′-TAA TGT CCA GGA AGT AGG TGA-3′; TNF-α forward, 5′-CTA CTG AAC TTC GGG GTG ATC-3′, and reverse, 5′-CAG TCG GCT AAA CGA TAG AGT-3′; GAPDH forward, 5′-GAT CAT CAG CAA TGC CTC CTC-3′, and reverse, 5′-TGT GGT CAT GAG TCC TTC CA-3′.

8. Immunoblot analysis

RAW 264.7 cells (5×10^6) were incubated with the indicated doses of proteins for the indicated times and then harvested. The cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 15 μg/ml leupeptin, 2 mM NaF and 2 mM $NaVO_4$) and the samples were loaded into SDS-acrylamide gels. The protein bands were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Pall Corporation, Ann Arbor, MI, U.S.A.). The membranes were blocked with blocking buffer [Tris-buffered saline (TBS) containing 5% nonfat dried milk] for 2 h and incubated with the indicated primary antibodies for 1 h on an orbital shaker. After washing three times with TBS containing 0.1%

Tween-20 (TBS-T), the membranes were incubated with secondary antibodies for 1 h. After washing three times with TBS-T, the membranes were developed using an ECL kit and then exposed to FUJI X-ray film (FUJI, Tokyo, JAPAN).

III. RESULTS

1. Induction of TNF-α secretion in RAW 264.7 cells by synuclein family proteins

To elucidate whether synuclein proteins activate macrophages, RAW 264.7 murine macrophage cells were first incubated with α-synuclein, and then the secretion of TNF-α in the cells was analyzed by ELISA. We used polymyxin B, an endotoxin inhibitor, to rule out the effect of contaminating endotoxin in the solution of recombinant proteins. When cells were incubated with 10 μg/ml polymixin B, 10 ng/ml LPS-induced TNF-α secretion was completed inhibited (Fig. 1A), indicating that polymixin B treatment could inhibit the contaminating endotoxin in recombinant proteins. Under these conditions, α-synuclein induced TNF-α secretion in RAW 264.7 cells in a dose-dependent manner (Fig. 1A). In a previous report, high doses of α-synuclein induced cell death of the neuroblastoma cell line SH-SY5Y [29]. To rule out the effect of cell death by α-synuclein, we also performed MTT assay, and observed that α-synuclein did not induce cell death of RAW 264.7 cells under the same conditions (Fig. 1B).

We next used β- and γ- synucleins, other members of the synuclein family, to clarify whether the effect of α-synuclein on TNF-α secretion in RAW 264.7 cells was a general property of synuclein family proteins. As shown in Fig. 2, β- and γ-synucleins, like α-synuclein, induced TNF-α secretion in RAW 264.7 cells in a dose-dependent manner (Fig. 2A-C). All synuclein proteins appeared to induce similar amounts of TNF-α secretion in RAW 264.7 cells. Furthermore, TNF-α mRNA expression was also increased by synuclein family proteins (Fig. 2D), suggesting that synuclein family proteins stimulated macrophages to induce TNF-α transcription.

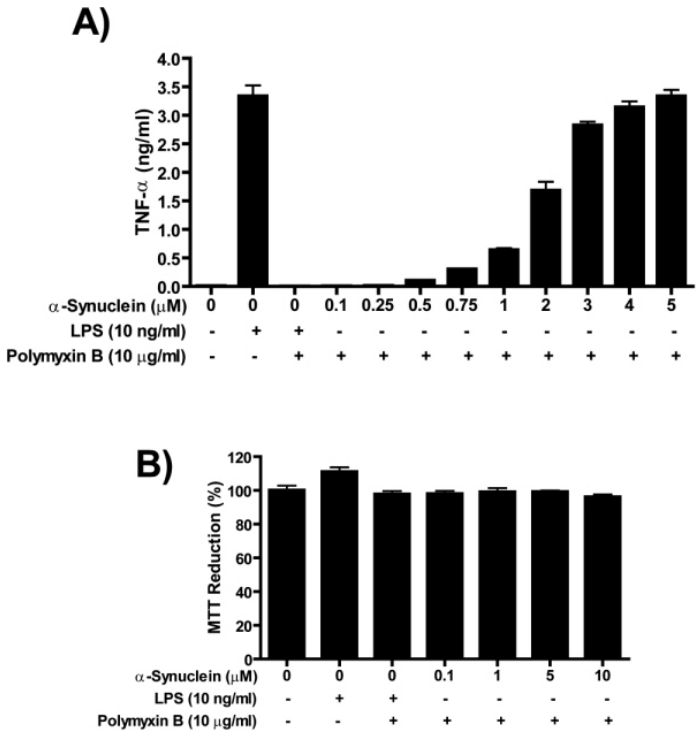

Fig. 1. α-synuclein induced TNF-α secretion in RAW 264.7 cells.

RAW 264.7 cells were incubated with the indicated amounts of α-synuclein for 18 h. Aggregated forms of α-synuclein were removed by gel-filtration chromatography before use. LPS was used for a positive control, and the effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment. (A) The amount of secreted TNF-α was measured in cell-free supernatants by ELISA. (B) Cytotoxicity of a-synuclein in Raw 264.7 cells was measured by MTT assay, as described in Materials and Methods. The data are presented as a mean of at least three independent experiments (mean±SD).

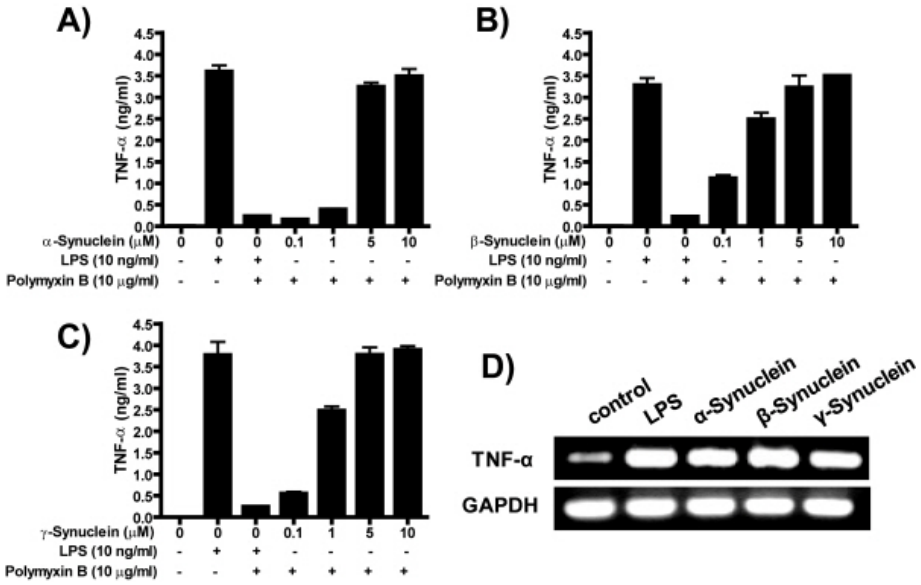

Fig. 2. Synuclein family proteins induced TNF-α secretion in RAW 264.7 cells.

RAW 264.7 cells were incubated with 0.1, 1, 5 or 10 μM of (A) α-, (B) β- or (C) γ-synuclein for 18 h. Aggregated forms of synuclein proteins were removed by gel-filtration chromatography before use. The effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment. The amount of secreted TNF-α was measured in cell-free supernatants by ELISA. (D) RAW 264.7 cells were treated with 1 μM of each synuclein family protein for 6 h. Total RNA was extracted from the treated cells and subjected to RT-PCR to quantify the mRNA transcripts of TNF-α. The internal control was set by GADPH. The data are presented as a mean of at least three independent experiments (mean±SD).

2. Effects of α-synuclein point mutants A30P, E46K and A53T on the induction of TNF-α secretion in RAW 264.7 cells

The three point mutants of α-synuclein (A30P, E46K and A53T) that are associated with a few cases of familial Parkinson's disease [12-14] have been thoroughly studied to determine the role of α-synuclein in the pathogenesis of Parkinson's disease. These mutant forms of α-synuclein appear to have different properties from the wild-type α-synuclein with respect to aggregation patterns, binding to lipid membranes and toxicity to cells [30-32]. We investigated whether these α-synuclein point mutants might function differently in the activation of macrophages. When RAW 264.7 cells were incubated with α-synuclein point mutants, each mutant protein induced more TNF-α secretion than wild type in the cells in a dose-dependent manner (Fig. 3A-C). Among these point mutants, A53T, in particular, appeared to induce more TNF-α secretion, even at low doses of synuclein treatment (Fig. 3D). These results suggest that these point mutations do not affect the mechanism by which wild type α-synuclein stimulates RAW 264.7 cells to secrete TNF-α.

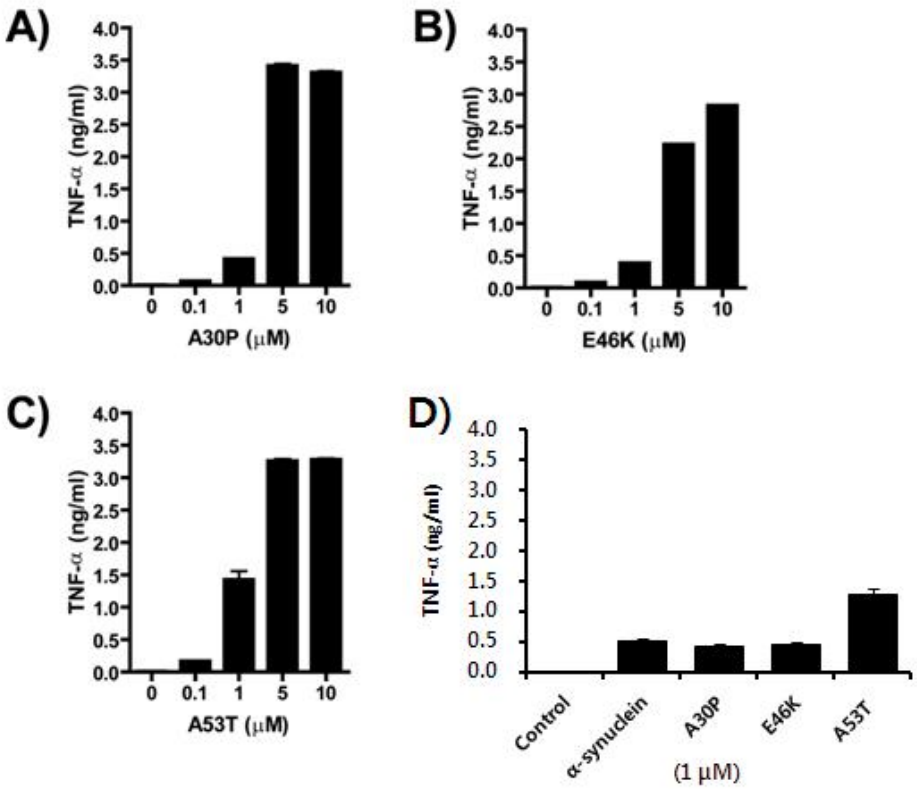

Fig. 3. α-synuclein point mutants A30P, A53T and E46K induced TNF-α secretion in RAW 264.7 cells.

RAW264.7 cells were incubated with the indicated amounts of (A) A30P, (B) E46K, (C) A53T mutant proteins and (D) various synuclein mutant proteins for 18 h. Aggregated forms of α-synuclein point mutants were removed by gel-filtration chromatography before use. LPS was used for a positive control, and the effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment. The amount of secreted TNF-α was measured in cell-free supernatants by ELISA. The data are presented as a mean of at least three independent experiments (mean±SD).

3. Effects of α-synuclein deletion mutants on the induction of TNF-α secretion in RAW 264.7 cells and primary macrophages

To elucidate the region of α-synuclein responsible for the induction of TNF-α secretion in RAW 264.7 cells, we used four deletion mutants: Syn1-60, containing the N-terminal region; ΔNAC, containing only the N- and C-terminal regions; Syn61-140, containing NAC and the C-terminal region and Syn96-140, containing only the C-terminal region of α-synuclein (Fig. 4A). When RAW 264.7 cells were incubated with these α-synuclein deletion mutants, ΔNAC and Syn61-140 induced the secretion of TNF-α in the cells (Fig. 4C, D), although the effect of syn 61-140 is less prominent than those of wild type and ΔNAC whereas, the other Syn1-60 and Syn96-140 did not significantly activate macrophages (Fig. 4B, E). Similar results were obtained when human primary macrophages were treated with these α-synuclein deletion mutants. (Fig. 4F). These results suggest that α-synuclein-induced TNF-α secretion requires the C-terminal domain of α-synuclein, as well as the N-terminal domain or NAC domain, which was identified as the internalization domain of α-synuclein in our previous report [28].

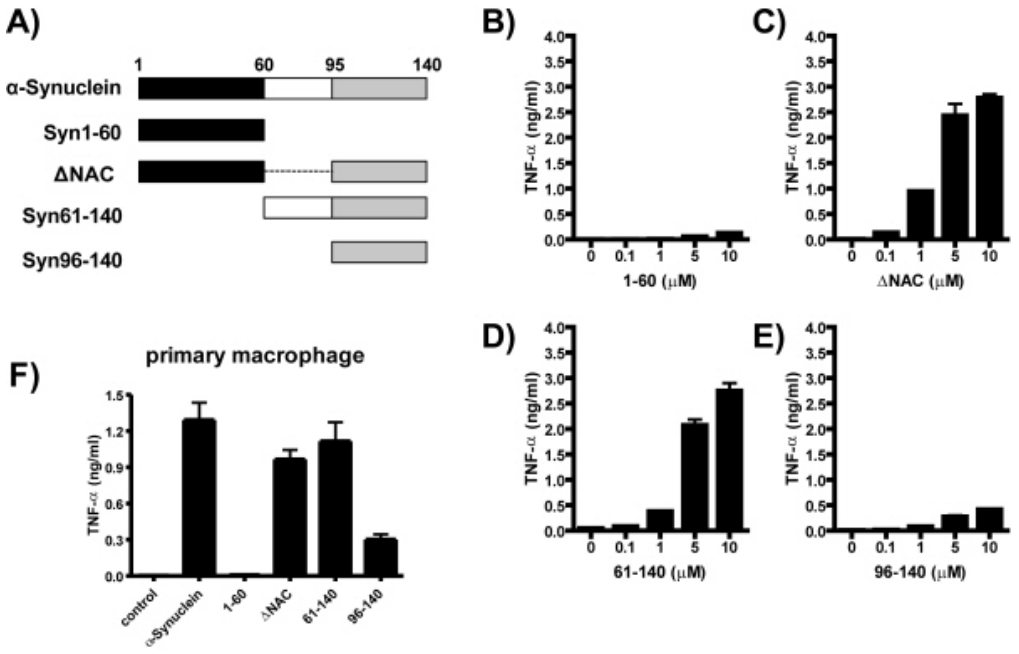

Fig. 4. Effects of α-synuclein deletion mutants Syn1-60, ΔNAC, Syn61-140 and Syn96-140 on the induction of TNF-α secretion in RAW 264.7 cells and Primary macrophage.

RAW 264.7 cells were incubated with the indicated amounts of (B) Syn1-60, (C) ΔNAC, (D) Syn61-140 and (E) Syn96-140 for 18 h. (F) Primary macrophage cells were incubated with 5 μM of α–synuclein, Syn1-60, ΔNAC, Syn61-140 and Syn96-140 for 24 h. Aggregated forms of α-synuclein and α-synuclein deletion mutants were removed by gel-filtration chromatography before use. The effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment, and the amount of secreted TNF-α was measured in cell-free supernatants by ELISA. The data are presented as a mean of at least three independent experiments (mean±SD).

4. Effect of synuclein proteins on the secretion of PGE_2 of RAW 264.7 cells and induction of COX2 and iNOS mRNA in RAW 264.7 cells

To further explore the effect of α-synuclein on the activation of macrophages, we analyzed PGE_2 secretion and mRNA expression of COX2 and iNOS, well-known inflammatory factors induced by macrophages. When RAW 264.7 cells were incubated with α-, β- and γ-synucleins, PGE_2 was secreted (Fig. 5A) in a dose-dependent manner (data not shown). We next investigated the effects of α-synuclein deletion mutants on the induction of PGE_2 secretion (Fig. 5B). When RAW 264.7 cells were incubated with 5 μM of each α-synuclein deletion mutant, wild-type α-synuclein and ΔNAC induced PGE_2 secretion in the cells. Syn61-140 induced less PGE_2 secretion than wild type α-synuclein, but the reduction was not statistically significant. Similar results were obtained when human primary macrophages were treated with these α-synuclein deletion mutants. As shown in Fig. 5C, ΔNAC and Syn61-140 induced the secretion of PGE_2 in the primary macrophages, while Syn1-60 and Syn96-140 did not significantly induced the secretion of PGE_2. These results were consistent with the observations of TNF-α secretion (Fig. 4).

Furthermore, when cells were incubated with α-, β- and γ- synucleins, all three synuclein proteins induced the expression of COX2 and iNOS mRNA, respectively (Fig. 5D).

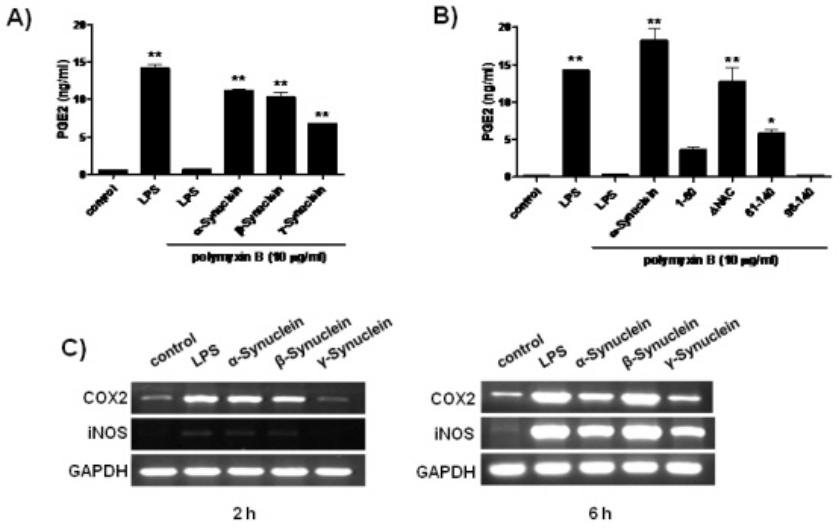

Fig. 5. Effects of synuclein proteins and α-synuclein deletion mutants on PGE_2 secretion and the expression of COX-2 and iNOS.

PGE_2 secretion was measured by ELISA after 18 h treatment with 5 μM of each (A) synuclein family protein or (B) α–synuclein deletion mutant in Raw 264.7 cells. (C) Primary macrophage cells were incubated with 5 μM of α–synuclein, Syn1-60, ΔNAC, Syn61-140 and Syn96-140 for 24 h. Aggregated forms of α-synuclein and α-synuclein deletion mutants were removed by gel-filtration chromatography before use. The effect of potentially contaminating endotoxin was neutralized by polymyxin B treatment, and the amount of secreted PGE_2 was measured in cell-free supernatants by ELISA. Statistical significance was evaluated using one-way ANOVA with post test. * $p < 0.05$, ** $p < 0.01$ against control. (D) RAW 264.7 cells were treated with 1 μM of each synuclein family protein for 2 h and 6 h. Total RNA was extracted from synuclein family protein-treated cells and subjected to RT-PCR to quantify the mRNA transcripts of COX-2 and iNOS. The internal control was set by GADPH. The data are presented as a mean of at least three independent experiments (mean±SD).

5. Phosphorylation of Extracellular signal-regulated kinases (ERK) in synuclein family-mediated RAW 264.7 cell activation

Macrophages can be activated by α-synuclein, and secrete TNF-α, IL-1β and other inflammatory molecules [25, 26]. Inflammatory molecules are induced via the activation of MAP kinase signaling pathways [33]. In particular, the ERK pathway is activated by α–synuclein treatment in microglia and astrocytes [25, 26]. We investigated whether MAP kinases were phospholylated by synuclein family proteins. RAW 264.7 cells were treated with 5 μM each of α-, β- or γ- synuclein for 30 min. Western blot analysis with specific antibodies against phosphorylated MAP kinases showed that total ERK, JNK and p-38 expression did not change after synuclein treatment (Fig. 6A, B and C). Among these kinases, the phospho-ERK signal was significantly enhanced after synuclein treatment compared with the control group (Fig. 6A). Unlike ERK phosphorylation, however, phospho-JNK and phospho-p-38 were not detected (Fig. 6B, C).

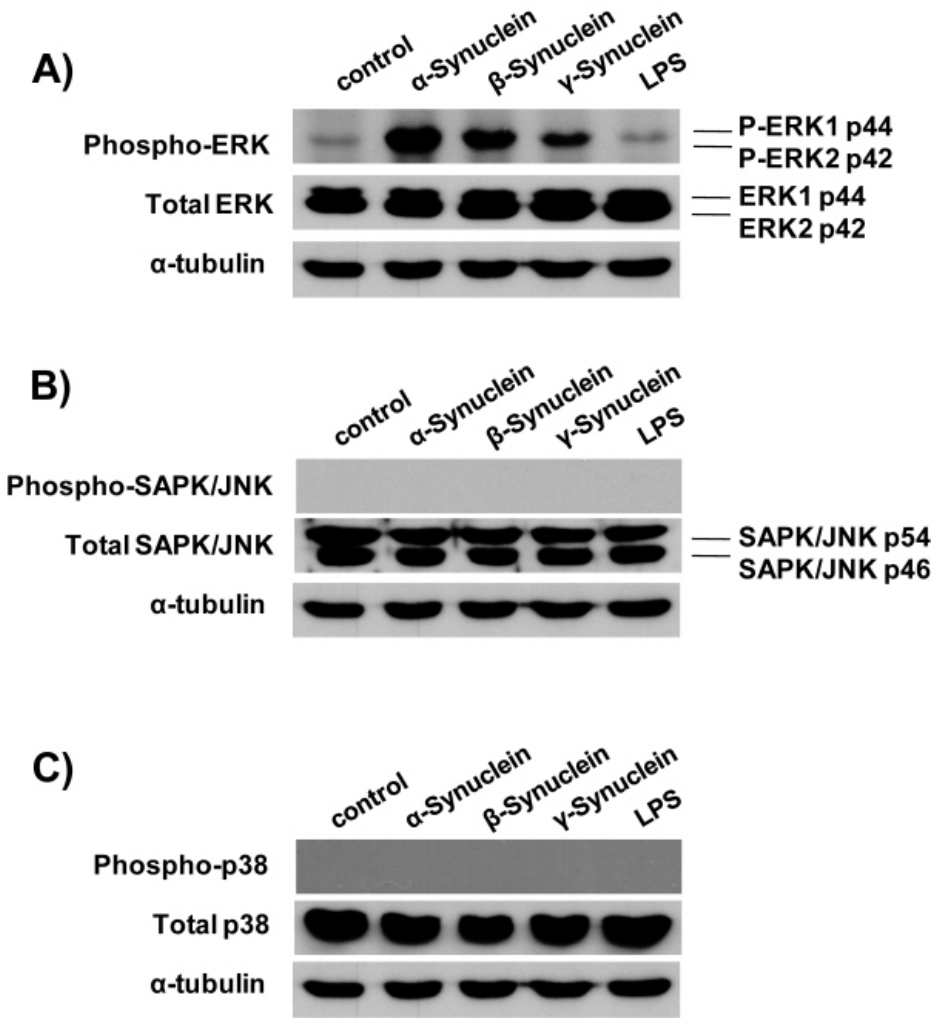

Fig. 6. Phosphorylation of MAP kinases by synuclein family proteins.

RAW 264.7 cells were treated with 5 μM of each synuclein family protein for 30 min. Total proteins were extracted from synuclein family protein-treated cells and 70 μg of each extraction were loaded onto SDS-acrylamide gels, followed by western blotting. The internal control was set by α-tubulin. (A) ERK and phospho-ERK, (B) JNK and phospho-JNK, (C) p38 and phospho- p38.

IV. DISCUSSION

Chronic inflammation is associated with a broad spectrum of neurodegenerative disorders, including Alzheimer's disease and PD [34-37]. The loss of dopaminergic neurons in PD is usually associated with glial reaction, particularly the activation of immunocompetent microglial cells. The conversion of glial cells associated with increased expression of cytokines and chemokines may trigger neurodegeneration in the CNS [34, 35]. Several studies report a marked increase of cytokine levels in the brain and CSF of patients with PD compared with age-matched controls [35, 38-40].

The physiological function of α-synuclein, originally identified as a neuronal cytoplasmic protein, has not been elucidated in detail. α-Synuclein is known to have chaperone activity [41, 42] and regulate dopaminergic neurotransmission and synaptic plasticity [2]. Interest in the role of extracellular α-synuclein has been spurred by recent findings [23, 25, 27, 29, 43], and several recent reports suggest that extracellular α-synuclein activates immunocompetent cells, which then induce neuronal degeneration [23, 25].

In the present study, we found that the treatment of α-synuclein in RAW 264.7 cells enhanced the production of TNF-α in protein level as well as mRNA level in a

dose-dependent manner, which is in agreement with previous work [25]. We also observed that α-synuclein enhanced TNF-α secretion in BV2 microglia cells (data not shown). Furthermore, α-synuclein enhanced PGE_2 secretion and COX2 and iNOS mRNA expression in RAW 264.7 cells, suggesting that α-synuclein activated RAW 264.7 cells. In addition, α-synuclein induced the phosphorylation of ERK1/2, but not JNK and p38. Results for β- and γ-synucleins were very similar to those for α-synuclein. Three point mutants associated with familial Parkinson's disease (A30P, E46K and A53T) also had stimulating effects. The degree of stimulation by synuclein family proteins was variable, suggesting that the cellular effect of α-synuclein is a general property of synuclein family proteins. Furthermore, polymyxin B treatment did not abolish the cellular effect of synuclein family proteins observed in this study, making it unlikely that results were due to bacterial contamination of samples.

α-Synuclein has recently been detected in extracellular biological fluids, including CSF and blood plasma in both healthy subjects and patients with Parkinson's disease [18-20], although it remains controversial whether the level of α-synuclein correlates with disease progression. In addition, γ-synuclein was also

recently found in CSF, and there is an elevation of both α- and γ-synuclein in CSF from elderly individuals with Alzheimer's disease, Lewy body disease and vascular dementia compared to normal controls [21]. Together with our findings, these data suggest that synuclein family proteins may be generally associated with neurodegeneration followed by immune system activation.

The N-terminal amphipathic regions of synuclein family members are well conserved, but the C-terminal acidic tails are very diverse in both size and sequence [2]. Based on the primary structural features, we speculated that the N-terminal region of α-synuclein could be important in RAW 264.7 cell activation. Interestingly, however, α-synuclein deletion mutants ΔNAC and 61-140 activated RAW 264.7 cells and primary macrophages, while 1-60 and 96-140 did not. The effect of ΔNAC is consistent with a report that the α-synuclein deletion mutant Δ71-82, which is unable to aggregate due to the lack of a corresponding middle hydrophobic region, effectively stimulates THP-1 cells [25]. We previously found that the N-terminal KTKEGV repeat of α-synuclein is essential for its penetration into cells [28]. Based on these findings, it may be that the 1-60 or NAC region of α-synuclein acts as a carrier

that transports α-synuclein into cells, and the region responsible for activating macrophages is the acidic C-terminal. Although the C-terminals of α-, β-, and γ-synucleins have low sequence homology, they do share the common property of containing many acidic amino acids. The C-terminal regions of α-, β-, and γ-synucleins are also capable of providing heat resistance to other proteins [44]. Although we could not exclude the possibility that α-synuclein may bind cell surface receptors and trasnduce signals for the activation of macrophages, our results suggested that penetration by α-synuclein may be essential for macrophage activation. The CD36 scavenger receptor is associated with α-synuclein-induced microglial activation. Furthermore, Mac-1 is also linked to mutant α-synuclein-induced microglial activation. It seems possible that α-synuclein could activate microglia via direct binding to these receptors. Further study is needed to elucidate the exact mechanism of how synuclein family proteins activate immunocompetent cells.

In summary, we showed that α-, β- and γ- synuclein activated macrophages. The penetration of α-synuclein into the cell may be needed to activate macrophages. The 1-60 or NAC region of α-synuclein may act as a carrier to transport α-synuclein into

cells, and the C-terminal acidic region may be responsible for activating macrophages by interacting with other effector molecule(s). In addition, the central NAC region may also contribute a little to transport syn 61-140 into cells, as has been demonstrated in a previous work[28]. Elucidating the mechanisms of immunocompetent cell activation induced by α-synuclein will provide new therapeutic targets for Parkinson's disease.

V. CONCLUSIONS

In this study we have shown the followings:

1. α-synuclein activated RAW 264.7 to secreted TNF-α and PGE_2.
2. α-synuclein did not effect to proliferation and death of RAW 264.7 cells.
3. Not only α-synuclein but also β- and γ-synuclein activated RAW 264.7 cells to secrete TNF- α and PGE_2.
4. Synuclein family induced TNF, COX2, and iNOS mRNA transcription levels in RAW 264.7 cells.
5. Synuclein point mutant such as A30P, E46K, and A53T could activate RAW 264.7 cells more than wild-type α-synuclein in RAW 264.7
6. Two α-synuclein deletion mutants, Syn ΔNAC and Syn 61-140, activated primary macrophages, as well as RAW264.7 cells, while Syn 1-60 and Syn 96-140 deletion mutants did not significantly activate them.
7. Macrophage activation by α-synuclein was accompanied by phosphorylation of ERK.

In conclusion, synuclein family proteins can activate macrophages, and that macrophage activation needs both the α-synuclein N-terminal domain for translocation into cells and the C-terminal domain for effector function. In addition, the macrophage activation by α-synuclein is accompanied by phosphorylation of ERK.

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ABSTRACT (IN KOREAN)

대식세포를 활성화 시키는 α-synuclein 단백질의 amino acid sequence motif 규명

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이 샛 별

만성 염증은 Parkinson's disease (PD)나 Alzheimer's disease와 같은 퇴행성 신경질환의 많은 부분과 연관되어 있다. 또한 활성화된 소교세포나 대식세포에서 분비되는 염증성 물질은 뇌신경세포의 사멸과 밀접한 관계가 있다. α-synuclein (Syn)은 PD 의 병인으로서 퇴행성 신경질환과도 연관되어 있다. 최근 연구에 의하면 α-synuclein은 소교세포를 활성화 시키며 도파민 작용성 퇴행성신경질환을 증가시킨다고 알려졌다. 그러나 α-synuclein에 의한 소교세포의 활성 기전은 아직까지 잘 알려져 있지 않았다. 이에 본

연구는 *in vitro*상에서 대식세포주 중의 하나인 RAW 264.7 세포와 일차 대식세포를 사용하여 α-synuclein 뿐만이 아니라 β-와 γ-synuclein도 대식세포를 활성화 시키는지를 확인하였다. 실험결과, 대식세포에 synuclein 단백질을 농도별로 처리하였을 때 분비되는 TNF-α와 PGE_2의 양이 처리해준 단백질의 농도에 의존적으로 증가하는 것을 확인하였다. 또한 synuclein 단백질은 COX2와 iNOS의 mRNA의 전사를 증가시켰다. α-synuclein 단백질의 결손형 돌연변이인 SynΔNAC와 Syn61-140은 RAW 264.7 세포와 일차 대식세포를 활성화 시켰고 syn61-140의 효과는 야생형이나 ΔNAC의 효과에는 다소 미치지 못하였다. 그러나 ΔNAC나 syn61-140과는 달리 Syn1-60과 Syn96-14 결손형 돌연변이체는 대식세포를 활성화 시키지 못했다. 또한 Synuclein 단백질의 점돌연변이체인 A30T, A53P, E46K는 α-synuclein 에 비해 대식세포를 더 강하게 활성화 시켰다. 마지막으로 α-synuclein에 의한 대식세포의 활성화는 ERK의 인산화를 동반한다는 것을 확인하였다. 이러한 결과는 Synuclein 단백질들이 대식세포를 활성화 시키는데 있어서 α-synuclein의 N-말단 부위는 세포 내로 들어가는데 필요하고, C-말단부위는 들어간 synuclein단백질이

대식세포를 활성화 시키는데 필요하며, N-말단부위가 결손 되어 있는 경우에는 세포 내로 synuclein 단백질이 들어가는데 있어서 NAC부위가 일정부분 이 역할을 수행할 수 있다는 것을 제시하고 있다.

핵심되는 말 : Parkinson's disease, synuclein, 대식세포, 염증, TNF-α, PGE_2

PUBLICATION LIST

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