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Phosphorylated IκBα is a component of Lewy body of Parkinson's disease

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Abstract

Ubiquitin is one of the major components of Lewy bodies (LB), the pathological hallmark of Parkinson's disease (PD). Here, we identified that a phosphorylated form of IκBα (pIκBα), an inhibitor of NF-κB, and $SCF^{\beta\text{-}TrCP}$, the ubiquitin ligase of pIκBα, are components of LB in brains of PD patients. In vitro studies identified those proteins in the ubiquitin- and α-synuclein (known as the major component of LB)-positive LB-like inclusions generated in dopaminergic SH-SY5Y cells treated with MG132, a proteasome inhibitor. Intriguingly, IκBα migration into such ubiquitinated inclusions in cells treated with MG132 was inhibited by a cell-permeable peptide known to block phosphorylation of IκBα, although this peptide did not influence cell viability under proteasomal inhibition. Our results indicate that phosphorylation of IκBα plays a role in the formation of IκBα-containing inclusions caused by proteasomal dysfunction, and that the generation of such inclusion is independent of cell death caused by impairment of proteasome.

Keywords: Parkinson's disease; Lewy bodies; Ubiquitin; Proteasome; IκBα; IκB-kinase

Parkinson's disease (PD) is one of the most common neurodegenerative disorders among the aged and its pathological hallmark is the preferential degeneration of dopaminergic neurons in the substantia nigra (SN) and the appearance of intracytoplasmic inclusions known as Lewy bodies (LB). The process of LB formation could provide important clues regarding the pathogenesis of PD because important proteins, such as α-synuclein (another familial PD gene product) and ubiquitin (Ub), are components of these inclusions [1,2].

The ubiquitin–proteasome pathway (UPP) is the major non-lysosomal degradation system for various proteins, such as short-lived, misfolded, and damaged polypeptides [3]. In this system, ubiquitin is conjugated to lysine residue of the target protein by a cascade of enzymatic reactions catalyzed by the E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes in an ATP-dependent manner, and polyubiquitination marks the proteins for degradation by the proteasome. Several lines of evidence suggest that derangements in the UPP play an important role in the pathogenesis of PD and describe inhibited hydrolytic activities of the proteasome in PD [4,5].

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On the other hand, the concentration of tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is increased in PD and this cytokine plays a role in the pathogenesis of PD [6,7]. TNF-α stimulates the multisubunit IκB-kinase (IKK), which is composed of IKKα, IKKβ and a non-catalytic regulatory component named NF-κB essential modifier (NEMO), which allows IκBα to be subsequently phosphorylated. The phosphorylated form, pIκBα, is polyubiquitinated by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase [8–10]. Subsequently, they are rapidly degraded by 26S proteasomes, and NF-κB enters the nucleus, binds to DNA, and activates transcription of target genes [11]. In this scenario, the nuclear translocation of NF-κB is masked by physical association with IκBα, resulting in retention of NF-κB in the cytoplasm.

Intriguingly, a previous report implicated the involvement of NF-κB in the LB of PD [12], but the role of IκBα in the pathogenesis of PD remains unclear. The aims of the present study were the following: (1) to determine the presence or absence of pIκBα and components of the $SCF^{\beta\text{-}TrCP}$ complex in LB of PD, (2) whether pIκBα is colocalized in the cytoplasmic inclusions formed in MG132-treated human dopaminergic neuroblastoma cells (SH-SY5Y), and (3) to determine the effect of inhibition of IκBα phosphorylation on the formation of cytoplasmic inclusions and viability of cells treated with a proteasome inhibitor.

Materials and methods

Human neuroblastoma cell lines. Human dopaminergic neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. To induce differentiation, the cells were treated with 10 μM retinoic acid (Sigma Chemical, St. Louis, MO) in the dark for 4–6 days as described previously [13–15].

Application of agents. TNF-α (R&D Systems, Minneapolis, MN) and MG132 (Peptide Institute, Osaka, Japan) were prepared at 10 μg/ml (in H_2O) and 10 mM (in dimethyl sulfoxide, DMSO) stock solutions, respectively. The specific antibodies used were rabbit anti-ubiquitin (Dako, Carpinteria, CA), mouse anti-ubiquitin 1510 (Chemicon International, Temecula, CA), anti-synuclein-1 (Transduction Laboratories, Lexington, KY), sheep anti-α-synuclein [16], anti-pIκBα (Ser32), and anti-IκBα antibodies (from Cell Signaling Technology, Beverly, MA, and Calbiochem, La Jolla, CA). Anti-NF-κB p65 (sc-372) and anti-β-TrCP (N-15) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Note that anti-IκBα antibody reacts with both phosphorylated and unphosphorylated forms of IκBα. Anti-ROC1 and Cul-1 antibodies were prepared as described previously [17]. The secondary antibodies used were goat anti-mouse IgG coupled with Alexa Fluor 488, goat anti-mouse IgG coupled with Alexa Fluor 594, anti-rabbit IgG coupled with Alexa Fluor 594 (Molecular Probes, Eugene, OR), and fluorescein goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

Immunohistochemistry. For the LB staining experiments using paraffin embedded samples, autopsied brains from seven patients with PD (age, 51–78 years), one patient with dementia with Lewy bodies (DLB, age, 64 years), and five control subjects (age, 20–65 years) were examined. The five control samples were obtained from patients free of neurological diseases and confirmed to have no neuropathological changes in sections of the substantia nigra pars compacta. Immunohistochemistry was performed as described previously [18].

Immunolabeling of isolated Lewy bodies. Immunomagnetic isolation and immunostaining of LB and Lewy neuritis from fresh frozen brains of patients with DLB were performed as described previously [19]. Smears were prepared on gelatin-coated glass slides, from homogenates, factions of each washing step, and LB-enriched Percoll fractions. The smears were air-dried overnight, fixed for 10 min in 4% formaldehyde–2% picric acid–0.1 M phosphate-buffered saline (pH 7.4), and then incubated for 10 min in 3% H_2O_2 in 50% methanol in Tris-buffered saline (TBS, pH 7.4). Following three rinses in 0.1 M TBS containing 0.05% sodium azide and 1 mM phenylmethylsulfonyl fluoride (PMSF) (TBS), the smears were incubated with 20% normal horse serum in TBS for 30 min to block non-specific antibody-binding sites, and incubated overnight with sheep antibody against α-synuclein and pIκBα, ROC1, Cul-1, and β-TrCP in TBS containing 0.5% bovine serum albumin. Control sections were stained by incubating the smears with TBS containing either or neither antibodies. Following three rinses in TBS, the smears were incubated for 1 h with donkey anti-sheep IgG or donkey anti-rabbit IgG conjugated with Cy2, Cy3 or Cy5 in TBS (all from Jackson Immunoresearch Laboratories, West Grove, PA), with the fluorochromes either singly or in combinations of Cy2/Cy3 or Cy2/Cy5.

Immunocytochemistry. For double-labeling immunofluorescence staining, fixed cells were permeabilized with 0.25% Triton X-100 for 20 min. The cells were blocked with 5% normal goat serum for 1 h, followed by incubation of antibodies to ubiquitin (Chemicon; diluted 1:100), pIκBα Ser32 (Cell Signaling; diluted 1:100), ROC1 (diluted 1:100), or Cullin-1 (diluted 1:100) for 1 h at room temperature. After washing in TBS, the cells were incubated in anti-mouse, anti-rabbit fluorochrome-linked secondary antibodies. After washing in TBS, the cells were covered with glass slides using mounting medium with 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei (Vector Laboratories). To assess the colocalization of ubiquitin and α-synuclein, we used anti-ubiquitin (Dako; diluted 1:100) and anti-synuclein-1 (diluted 1:100) antibodies. Signal was observed under a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, NY).

Western blotting. Neuronally differentiated SH-SY5Y cells were treated for 24 h with 10 μM MG132 or 20 ng/ml TNF-α. The cells were simultaneously preincubated for 2 h with 10 μM MG132, followed by treatment for 22 h with 10 μM MG132 in the presence of 20 ng/ml TNF-α. The cells were collected and washed in ice-cold phosphate-buffered saline (PBS) and lysis buffer as described previously [20]. Detergent-insoluble material was pelleted by centrifugation at 100,000*g* for 20 min and resuspended in 50 mM Tris–HCl (pH 7.4) and 6 M urea. Equal amounts of protein from both fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% or 10–20%) and transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in 5% non-fat milk for 1 h at room temperature, and incubated at 4 °C with specific antibodies.

Treatment of IKK inhibiting peptide. Recent studies have reported that an NH_2-terminal α-helical region of NEMO associates with a hexapeptide sequence within the extreme carboxyl terminus of IKKβ and IKKα, termed NEMO-binding domain (NBD). Importantly, a short cell-permeable peptide spanning the IKKβ NBD was found to disrupt the association of NEMO with IKKβ and blocked the activity of IKK [21]. We synthesized two peptides at Juntendo University School of Medicine, a functional wild-type NBD with a sequence derived from the Antennapedia homeodomain that mediates membrane translocation without loss of cell viability, and a negative control mutant NBD, as described previously [21–24]. To determine how inhibition of phosphorylation of IκBα affects the formation of such inclusions, the differentiated SH-SY5Y cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 10 μM MG132 in the presence of 40 μM of each NBD peptide. The cells were simultaneously preincubated for 3 h

with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. These cells with ubiquitin-positive inclusions were co-stained with antibodies against pIκBα or IκBα.

Cell viability assay. Cell viability assay was performed using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt assay, as described previously [25]. The differentiated SH-SY5Y cells were plated on a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2. The cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 40 μM of each NBD peptide in the presence or absence of 10 μM MG132. The cells were simultaneously preincubated for 3 h with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. The live cell count was assayed using Cell Counting Kit-8 according to the instructions provided by Doujin (Cell Counting Kit-8; Kumamoto, Japan).

Statistical analysis. All data are expressed as means ± SEM. Comparisons between groups were performed using analysis of variance (Tukey's multiple *t* test).

A p value <0.05 indicated statistically significant differences.

Results

Phosphorylated IκBα and $SCF^{\beta\text{-}TrCP}$ complex are novel components of Lewy bodies

We first examined whether LB contain pIκBα and the components of $SCF^{\beta\text{-}TrCP}$ complex, which are major downstream components of the TNF-α signaling pathway. Immunohistochemical analysis revealed that anti-pIκBα and ROC1 antibodies predominantly recognized the LB in PD cases (Figs. 1A and B). Immunostaining with anti-NF-κB p65 antibody also showed the staining of LB (Fig. 1C). Anti-pIκBα and anti-ROC1 signals were strongly present in the halo region of LB, and the anti-NF-κB-p65 signal was present in the core region. In contrast, such immunoreactivities for pIκBα ROC1 and NF-κB were not observed in the control brains and when the primary antibody was omitted in PD and DLB brains (data not shown).

In the next step, the isolated LB were used to investigate whether these proteins are associated with LB. Confocal laser-scanning microscopic examination of sections prepared from freshly isolated LB from postmortem brains of DLB showed immunoreactivities for pIκBα, ROC1, Cul-1, and β-TrCP (Figs. 1D, G, J, and M). LB were identifiable by their strong α-synuclein staining in smears of isolated LB from DLB cortex (Figs. 1E, H, K, and N), but not in sections from a normal control cortex (data not shown). These immunoreactivities for the indicated antibodies in LB were distributed across or sometimes more concentrated in the central region of LB (Figs. 1F, I, L, and O). Counting of α-synuclein-positive LB indicated that 80–90% of the cortical LB ($n = 300$, pooled from three DLB cases) were also positive for ROC1 and Cul-1. A similar staining pattern was also observed in LB isolated from the substantia nigra (SN) of PD (data not shown).

Localization of pIκBα, ROC1, and Cul-1 in cytoplasmic inclusions of SH-SY5Y cells

As a model for the formation of cytoplasmic inclusions, we used SH-SY5Y cell lines treated with MG132 [26]. Localization of pIκBα, ROC1, and Cul-1 was investigated after the addition of 10 μM MG132 for 24 h in differentiated SH-SY5Y cells. Proteasomal dysfunction caused typical cytoplasmic inclusions that were stained with anti-ubiquitin (Ub) antibodies, and interestingly many, if not all, ubiquitinated inclusions were also positive for pIκBα, ROC1, and Cul-1 (Figs. 2A–C). Under normal conditions without MG132, the cells displayed low-level cytoplasmic staining for the indicated proteins (data not shown). Although we examined the effect of TNF-α on the formation of the inclusions, no inclusions that contained ubiquitin and pIκBα were observed after treatment with TNF-α alone. In addition, the effect of simultaneous treatment with TNF-α and MG132 was not significantly different from the results of MG132 treatment alone (data not shown).

We next examined whether these cells also contained α-synuclein in such cytoplasmic ubiquitinated inclusions. Following proteasomal inhibition with 10 μM MG132, some of the ubiquitinated cytoplasmic inclusions also exhibited α-synuclein immunoreactivity (Fig. 2D). Moreover, we examined whether pIκBα and components of the SCF complex colocalize with α-synuclein in the presence of 10 μM MG132. The α-synuclein-positive inclusions were also immunoreactive for pIκBα ROC1 and Cul-1 following treatment with 10 μM MG132 (Figs. 2E–G). The proportion of cells treated with 10 μM MG132 that contained aggregates immunoreactive for both pIκBα and α-synuclein was 7.98 ± 1.14%. In contrast, the proportion of 10 μM MG132-treated cells containing inclusions positive for both pIκBα and ubiquitin was 23.19 ± 3.84%, suggesting the relative low frequency of α-synuclein/pIκBα-containing inclusions (see Fig. 5B). Inclusions containing only ubiquitin, α-synuclein, or pIκBα were also noted, and their size was also comparatively heterogeneous (data not shown).

Inhibition of proteasomes increases phosphorylated IκBα level in SH-SY5Y cells

We examined the migration pattern of endogenous ubiquitin or pIκBα by SDS–PAGE in differentiated SH-SY5Y cells following proteasomal inhibition with MG132 and/or TNF-α for 24 h. Cells were treated as indicated in Fig. 3, and then the resulting cell extracts were separated into detergent-soluble and detergent-insoluble fractions. Treatment with 10 μM MG132 resulted in accumulation of high-molecular weight ubiquitin–protein conjugates particularly within the insoluble fractions but not in TNF-α alone and control

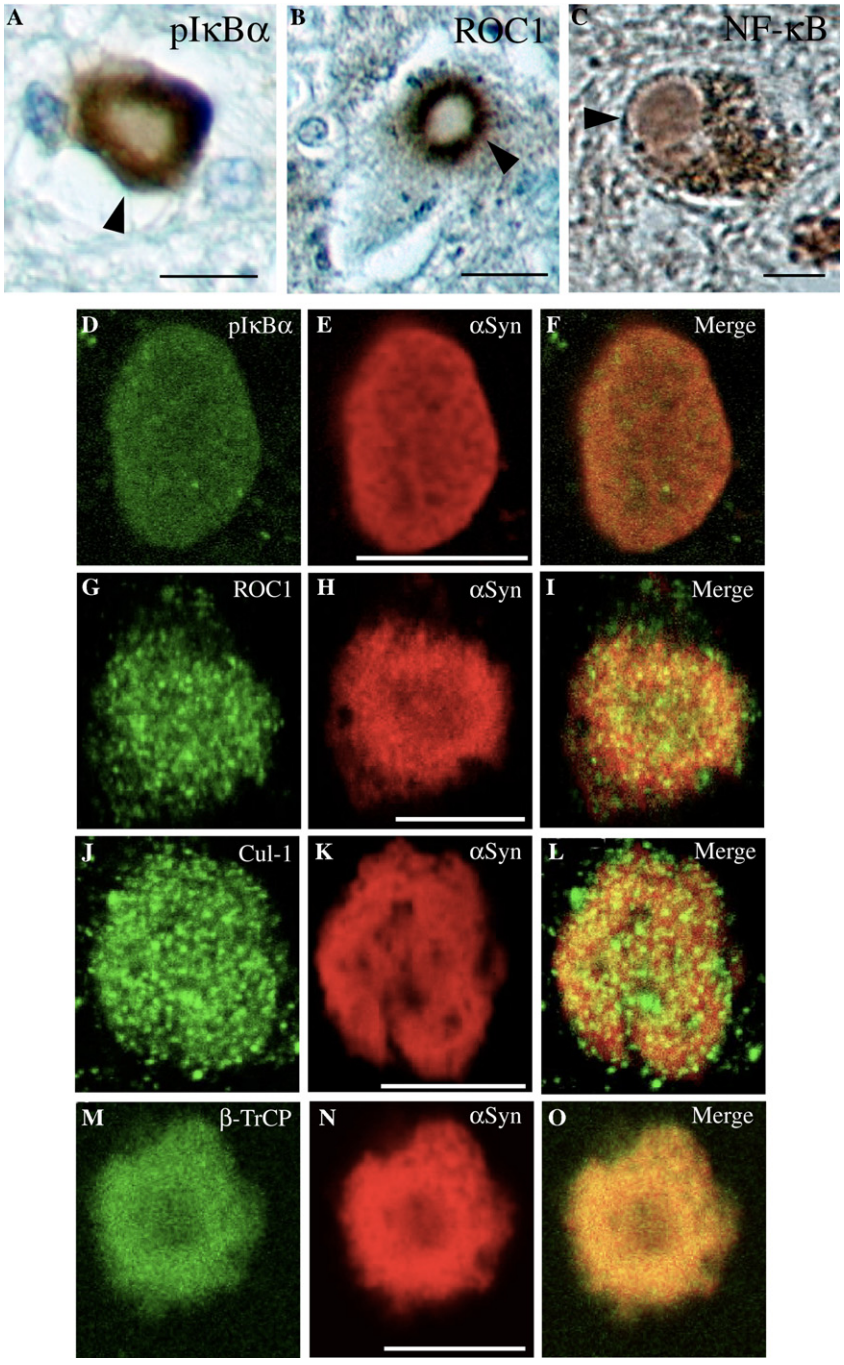

Fig. 1. Identification of phosphorylated IκBα and components of $SCF^{\beta\text{-}TrCP}$ in Lewy bodies. (Upper panel) Paraffin sections of autopsied human brain samples with PD were immunostained with antibodies against pIκBα (A), ROC1 (B), and NF-κB p65 (C). Lewy bodies are marked by arrowheads. Scale bars = 20 μm (A–C). (Lower panel) Colocalization of α-synuclein (αSyn), pIκBα, and components of $SCF^{\beta\text{-}TrCP}$ in isolated LB from DLB (Dementia with LB) cases. LB were identified by α-synuclein staining. Each preparation was doubly stained with sheep anti-α-synuclein (E, H, K, and N) and various antibodies against pIκBα (D), ROC1 (G), Cul-1 (J), and β-TrCP (M), and analyzed with a laser-scanning confocal microscope. Panels (F, I, L, and O) at right correspond to merged images; yellow-colored structures indicate colocalization. Scale bars = 10 μm (D–O).

cells (Fig. 3A). Unexpectedly, the effect of TNF-α was very weak in SH-SY5Y cells, because no massive reduction of IκBα was observed upon treatment with TNF-α for 1, 12, or 24 h (Fig. 3C and data not shown). This finding was in marked contrast to the almost complete disappearance of IκBα in HeLa cells treated with 20 ng/ml TNF-α within 1 h (data not shown). However, TNF-α significantly increased the pIκBα level (Fig. 3B), indicating the existence of TNF-α response to a lesser extent in SH-SY5Y cells. It is of note that MG132 alone

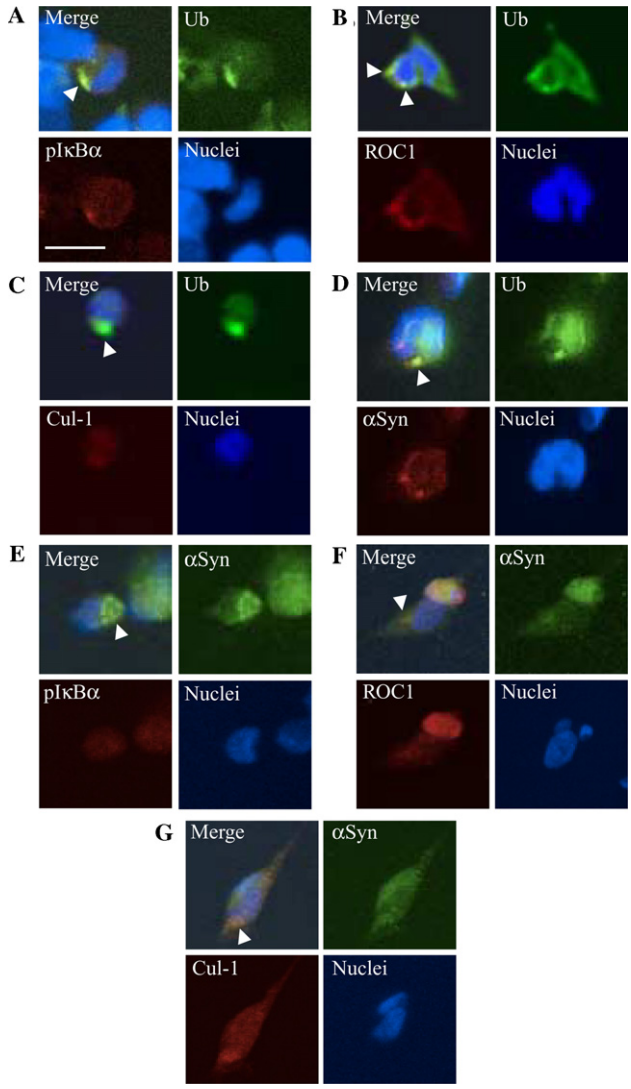

Fig. 2. Proteasomal inhibition leads to formation of pIκBα-positive cytoplasmic inclusions in SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with 10 μM MG132 for 24 h, fixed and then double-stained with various combinations of antibodies as indicated. (A–D) Cytoplasmic inclusions positive for ubiquitin (Ub) were co-stained for pIκBα (A), ROC1 (B), Cul-1 (C), and α-synuclein (D). Arrowheads indicate the inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. (E–G) α-Synuclein (αSyn)-positive cytoplasmic inclusions were co-stained for pIκBα (E), ROC1 (F), and Cul-1 (G). Regions of overlap between α-synuclein (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. Scale bar = 10 μm.

increased the pIκBα level in the cells (Fig. 3B), although additive effects of TNF-α and MG132 were not observed for phosphorylation of IκBα. Intriguingly, when detergent-soluble and -insoluble fractions were immunoblotted with anti-pIκBα or anti-IκBα antibody, both proteins were clearly detected in the detergent-insoluble fraction after treatment with 10 μM MG132 but not in TNF-α alone and control cells (Figs. 3B and C). In addition, simultaneous treatment with TNF-α and MG132 had no significant effects in comparison with MG132

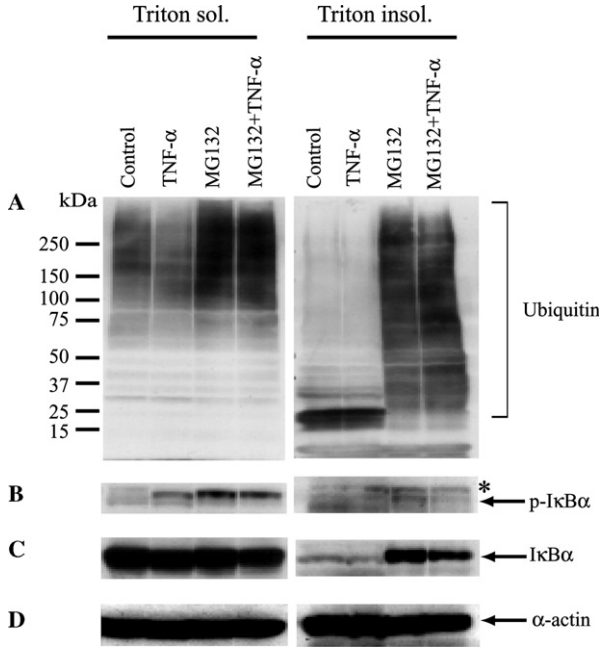

Fig. 3. Inhibition of the proteasome results in accumulation of pIκBα within the detergent-insoluble fraction of SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 and/or 20 ng/ml TNF-α, and the cell lysates were processed for Western blotting, as described in Materials and methods. The protein was blotted onto PVDF membranes and probed with antibodies against ubiquitin (A), pIκBα (B), and IκBα (C). Note that anti-IκBα antibody reacted both phosphorylated and unphosphorylated forms. α-Actin served as a loading control (D). Asterisk indicates a non-specific band.

treatment alone. Thus, it is clear that IκBα, perhaps its phosphorylated form, is incorporated into the detergent-insoluble fraction under the conditions of proteasome inhibition.

The NBD peptide inhibits pIκBα entry into cytoplasmic ubiquitin-positive inclusions

The presence of pIκBα in LB of autopsied brains of PD patients and ubiquitinated inclusions in SH-SY5Y in the present study led us to examine whether inhibition of IKK, which phosphorylates IκBα, alters the processes of inclusion formation and cell death. First, we determined the optimal concentration of the cell-permeable NBD peptide, which is known to block the activity of IKK. To study the effect of NBD, SH-SY5Y cells were pre-treated with various concentrations of wild-type NBD for 3 h and then stimulated by 20 ng/ml TNF-α. In the present study, we used 40 μM NBD as the optimal concentration to block phosphorylation of IκBα. We also examined the effect of high concentrations of the NBD peptide (about 1000 μM), as described previously [21], but peptide toxicity was observed in our cell lines.

We next treated the cells with MG132 in the presence or absence of NBD peptide and then performed double

staining using antibodies for pIκBα, IκBα, and ubiquitin. Ubiquitinated inclusions containing pIκBα were identified in cells treated with MG132 alone or with MG132 in the presence of mutant NBD lacking inhibitory activity for IKK. On the other hand, while ubiquitinated inclusions were observed in cells treated with MG132 in the presence of wild-type NBD, only a few cells contained ubiquitinated inclusions positive for pIκBα (Fig. 4A). In addition, the use of an antibody for IκBα in the presence of wild-type NBD was also associated with reduced number of cells with ubiquitinated inclusions positive for IκBα, compared with those treated with MG132 or MG132 in the presence of mutant NBD (Fig. 4B), indicating that phosphorylation of IκBα may be required for its incorporation into cytoplasmic inclusions generated by proteasome inhibition.

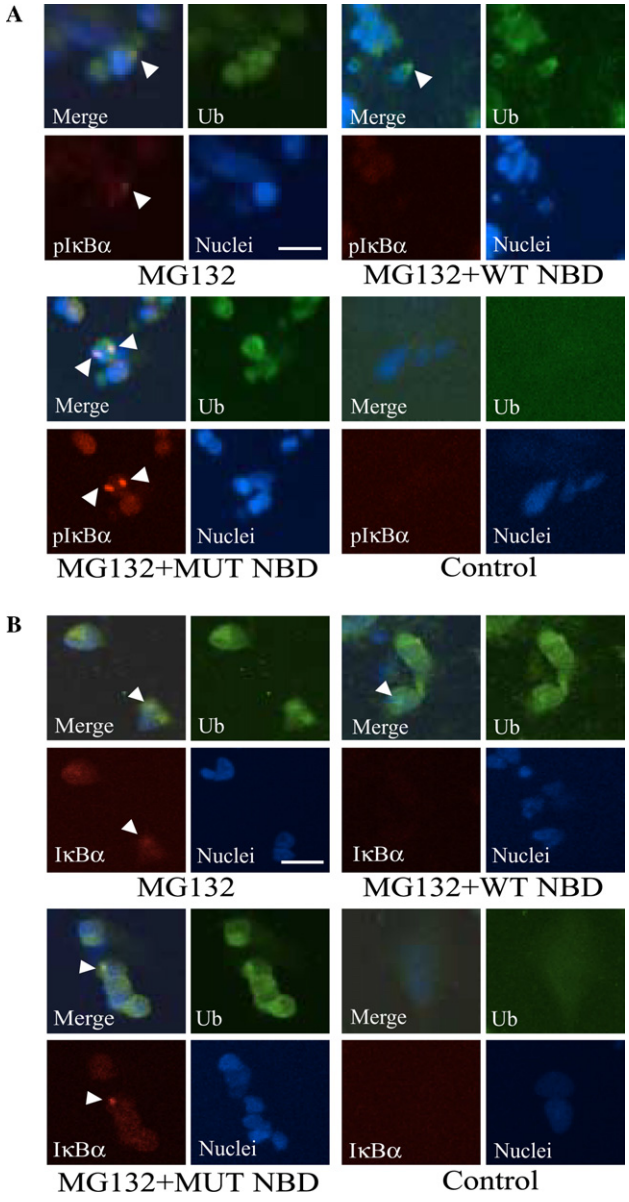

Fig. 4. Wild-type NBD decreases pIκBα level within the ubiquitinated inclusions in SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 alone, or with 10 μM MG132 in the presence of 40 μM of either wild-type (WT NBD) or mutant NBD peptide (MUT NBD) as indicated in Materials and methods. Cells with ubiquitinated inclusions were co-stained with pIκBα (A) and IκBα (B). Arrowheads indicate the ubiquitinated inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) are shown in yellow color. Scale bar = 20 μm.

We then counted the number of cells with aggregated immunoreactivity for both ubiquitin and pIκBα antibodies under basal condition and following treatment with 10 μM MG132 with or without NBD peptide. It is worth noting that whereas approximately 50% of total cells contained ubiquitin-positive inclusions, pIκBα-positive inclusions were below 25% (Figs. 5A and B), suggesting that pIκBα is not incorporated into all inclusions. Wild-type NBD significantly decreased the number of cells with ubiquitinated inclusions (Fig. 5A, $p < 0.05$), and cells with cytoplasmic inclusions positive for pIκBα and ubiquitin, compared with cells treated with MG132 alone (Fig. 5B, $p < 0.001$). In comparison, mutant NBD did not show the same effects on phosphorylation of IκBα as wild-type NBD. Finally, we examined the toxicity of 10 μM MG132 on these cell lines. Treatment with 10 μM MG132 reduced cell viability to $37.84 \pm 1.46\%$. In contrast, wild-type NBD did not influence cell viability under proteasomal inhibition (Fig. 5C).

Discussion

The appearance of LB in SN is a prominent feature in PD, but the pathogenic role of such inclusions remains elusive. In this study, we identified novel components including pIκBα and components of $SCF^{\beta\text{-}TrCP}$ ligase in LB. To date, several studies have reported that the UPP-related proteins (such as ubiquitin, the 20S proteasome subunit, and HSP70) are localized in LB of PD [27,28]. These findings indicate that there appears to be an important correlation between some pathological alteration in UPP and the formation of LB in PD. In this regard, the pathogenic nature of proteasomal dysfunction has been studied in experimental models using a proteasome inhibitor. It has been demonstrated that inhibition of proteasomal function induces the formation of cytoplasmic inclusions immunoreactive for ubiquitin and α-synuclein in PC12 cells and mesencephalic cultures [20,29]. These observations suggest that proteasomal dysfunction is associated with the development of cytoplasmic inclusions that may have features similar to those of LB, in terms of containing two proteins; i.e., α-synuclein and ubiquitin, described as the major components of LB [2].

pIκBα and SCF ligase are also involved in UPP-related proteins, and these molecules have not been adequately studied in PD. Therefore, to explore how these

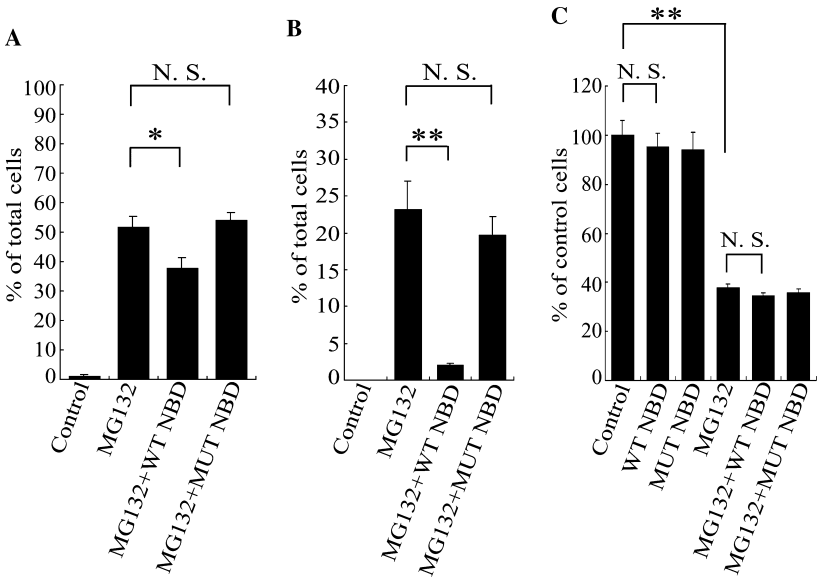

Fig. 5. Effects of NBD on formation of pIκBα-containing inclusions and cell death caused by proteasomal inhibition. After the cells were treated as explained in Fig. 4, the proportions of cells with cytoplasmic inclusions were determined. (A) The proportion of cells with ubiquitin-positive inclusions was calculated relative to total cells. (B) The proportion of cells with ubiquitinated inclusions containing pIκBα was calculated relative to total cells. In each experiment, 10 fields of 50 cells were counted. Similar effects of NBD were seen in two or more independent experiments. Data are means ± SEM. $*p < 0.05$; $**p < 0.001$ for differences between cell lines (Tukey's multiple t test). NS, not significant. (C) Cell viability was assessed as described in Materials and methods, and is expressed as the percentage of untreated cells. Similar results were seen in three independent experiments. Values are means ± SEM, each $n = 8$. $**p < 0.001$ for differences between cell lines (Tukey's multiple t test).

molecules are present in LB would be important in considering the process of LB formation. In our cell culture model, inhibition of normal proteasomal function by MG132 also induced the formation of ubiquitinated cytoplasmic inclusions containing α-synuclein, and this finding is consistent with previous reports [20,29], as described above. Intriguingly, our results showed that these inclusions were positive for pIκBα and some components of its ligase that are found in the LB. These findings suggest that the existence of pIκBα in LB is more likely and proteasomal dysfunction is an important factor in the formation of cytoplasmic inclusions.

Using SDS–PAGE analysis of detergent-soluble and -insoluble fractions, we found high-molecular weight ubiquitinated proteins particularly in the detergent-insoluble fraction, and pIκBα in the insoluble fraction following proteasomal inhibition with MG132. In contrast, after incubation with TNF-α alone, neither high-molecular weight ubiquitinated bands nor pIκBα was detected in the insoluble fractions, and cytoplasmic inclusions containing ubiquitin and pIκBα were not observed. These findings suggest that phosphorylation of IκBα alone is insufficient for the formation of cytoplasmic inclusions, and there appears to be a strong causal link between the accumulation of poorly degraded proteins, resulting from proteasomal dysfunction, and the formation of cytoplasmic inclusions.

We also showed that the presence of pIκBα in the ubiquitinated inclusions was markedly inhibited by a specific IKK inhibitor, under the conditions of MG132 treatment. This finding also supports the above-mentioned data that pIκBα is involved in the cytoplasmic inclusions resulting from proteasomal inhibition in our SH-SY5Y cells. In addition, this finding provides us a further possibility. In some neurodegenerative disorders, the ubiquitin-positive inclusions are considered to involve the ubiquitin–protein conjugates [28,30]. However, it is not clear which types of proteins are directly polyubiquitinated in LB. IκBα is phosphorylated by IKK, and pIκBα is polyubiquitinated by the $SCF^{\beta\text{-}TrCP}$, then degraded by the 26S proteasome. Thus, it is conceivable that once phosphorylation of IκBα is inhibited, neither polyubiquitination after its phosphorylation nor accumulation of IκBα into inclusion bodies is observed. We demonstrated that wild-type NBD peptide reduced the proportion of not only ubiquitin-positive inclusions, but also ubiquitinated inclusions containing pIκBα. Based on our finding, it is possible that the polyubiquitination of pIκBα resulting from proteasomal dysfunction triggers its entry into ubiquitinated cytoplasmic inclusions.

It is still not clear whether LB are cytoprotective or cytotoxic for neurons in the SN of PD. Recent studies suggest that the formation of protein aggregates or intracellular inclusions may be beneficial for cell survival rather than enhance cell death [31,32]. In the present study, exposure to MG132 alone or MG132 in the presence of wild-type NBD peptide did not alter cell viability whereas the same conditions decreased the ubiquitinated cytoplasmic inclusions. This finding at least supports the

conclusion of the above studies [31,32], i.e., the formation of cytoplasmic inclusions is not a toxic response against cell survival. Viewed from a different angle, our finding may suggest that inclusion bodies formed following proteasomal inhibition are independent of cell death.

In conclusion, we demonstrated the presence of pIκBα in LB of PD, and that similar inclusion bodies are produced in the presence of significant proteasomal dysfunction in cultured cells. Our observations in cultured cells may reflect, at least in part, the formation of LB in dopaminergic neurons of PD.

Acknowledgments

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