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Research Paper

Tumor necrosis factor receptor-associated factor 6 interaction with alpha-synuclein enhances cell death through the Nuclear Factor-kB pathway

Lidia M. Yshii [a], Adriana O. Manfiolli [b], Alexandre Denadai-Souza [c], Paula F. Kinoshita [a], Marcelo D. Gomes [b], Cristoforo Scavone [a,*]

[a] Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, São Paulo, 05508-900, Brazil
[b] Department of Biochemistry and Immunology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, 14049-900, Brazil
[c] Section of Experimental Endocrinology, Department of Pharmacology, Federal University of Sao Paulo, São Paulo, 04044-020, Brazil

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ABSTRACT

Background: Parkinson's disease (PD) is a neurodegenerative disease characterized by intracellular inclusions named Lewy bodies (LB), and alpha-synuclein (asyn) is the major component of these protein aggregates. The precise physiological and pathological roles of asyn are not fully understood. Nevertheless, asyn present in LB is ubiquitinated but fails to reach the 26S proteasome. The mutation A30 P is related to an aggressive and early-onset form of PD. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase, and it interacts and ubiquitinates the asyn in atypical chains (lysine K6, K27, K29, and K33). Methods: Here, we investigated the role of TRAF6 interaction with asyn and the involvement of nuclear factor κB (NF-κB), a key transcription factor in pro-inflammatory signaling pathway activation.
Results and Conclusion: We demonstrated that TRAF6 binds to both WT and the mutant form A30 P asyn in an SH-SY5Y cell model. Additionally, the interaction between TRAF6 and WT asyn induced an increase in the activation of NF-κB, leading to changes in *TNF, IL-1β* and *IL-10* levels and culminating in reduced cell viability. Interestingly, the activation of NF-κB and gene regulation were not found in A30 P asyn. These data point to a novel role of TRAF6 in the pathophysiology of PD.

Introduction

Alpha-synuclein (asyn, gene: *SNCA*) is a 140 amino acid presynaptic protein and is the major component of intracellular inclusions called Lewy Bodies (LB), which are present in dopaminergic neurons (Spillantini et al., 1997).

The physiological and pathological function of asyn is not fully understood, but several studies point towards the crucial involvement of asyn accumulation and misfolding in Parkinson's disease (PD) (Winner et al., 2011). Additionally, *SNCA* mutations, such as A30 P, A53 T, and E46 K, are the main characteristics of familial PD and are related to early onset of the disease (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Mice overexpressing A30 P asyn ((Thy-1)-h[A30 P] α-syn tg mice) present asyn aggregation, and asyn accumulates in neuronal cell bodies and neurites (Kahle et al., 2000). These animals showed motor, cognitive impairment, and GFAP immunoreactivity in the areas of asyn accumulation (Ekmark-Lewén et al., 2018). A30 P asyn mutation also impairs autophagic flux and neurogenesis by decreasing dopamine levels (Lei et al., 2019).

The proteins present in LB are identified for degradation, but they are not removed efficiently (Mayer et al., 1996; Ross and Poirier, 2004). Likewise, the asyn present in LB is ubiquitinated, but it seems that it fails to reach the 26S proteasome (Anderson et al., 2006). Furthermore, there are other mutations linked to familial PD, for example, in Parkin, an E3 ubiquitin ligase (Lohmann et al., 2009).

Another E3 ubiquitin ligase is a tumor necrosis factor receptor-

Abbreviations: PD, Parkinson's disease; LB, Lewy bodies; asyn, alpha-synuclein; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor κB; EMSA, Electrophoretic Mobility Shift Assay; CHIP, carboxyl terminus of Hsp70-interaction protein; SIAH, seven in absentia homolog.

* Corresponding author at: Department of Pharmacology, Institute of Biomedical Science ICB-1, Avenida Professor Lineu Prestes, 1524, University of São Paulo, São Paulo, 05508-900, Brazil.
E-mail address: criscavone@usp.br (C. Scavone).

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associated factor 6 (TRAF6) (Inoue et al., 2000; Bradley and Pober, 2001). The polyubiquitination of lysine K63 by TRAF6 plays a fundamental role in the signaling pathway involved in nuclear factor-κB (NF-κB) activation (Chen, 2005). Recently, Zucchelli et al. (2010) showed that TRAF6 interacts with and ubiquitinates the asyn in atypical chains (lysines K6, K27, K29, and K33). Other pieces of evidence suggest a role for TRAF6 in neurodegeneration, including, for example, the colocalization with tau and huntingtin protein in the brains of patients with Alzheimer's (Babu et al., 2006) and Huntington's disease (Zucchelli et al., 2011), respectively.

Non-conventional ubiquitination can be associated with a variety of cell functions, such as the degradation of proteins and the regulation of proteasome activity. Therefore, it represents a signaling pathway that could play an essential role in the pathogenesis of PD. TRAF6 is also vital to mediating immune receptor signaling, such as TNFRs, and modulates NF-κB activity (Shi and Sun, 2018). Neuroinflammation is a relevant component in PD. Microglial activation increases cytokine levels, and increased activation of NF-κB has been described in PD (Shi and Sun, 2018; Hunot et al., 1997; Ghosh et al., 2007).

This study aimed to investigate the role of TRAF6 interaction with asyn and the involvement of NF-κB, a key transcription factor in pro-inflammatory signaling pathway activation (Lawrence, 2009). Here, we show that TRAF6 binds to both WT and the mutant form A30 P asyn in an SH-SY5Y cell model. Furthermore, the interaction between TRAF6 and WT asyn induced an increase in the activation of NF-κB, leading to changes in TNF, IL-1β, and IL-10 levels, culminating in reduced cell viability. These data point to a possible novel role of TRAF6 function under normal and pathological conditions.

Experimental Procedures

Plasmids

WT or A30 P asyn cDNA was cloned into the pCS2-MT plasmid (Addgene, Cambridge, MA USA). cDNAs coding for WT and A30 P human α-syn (GenBank no. NM_000345.2) were kindly provided by Dr. Pamela McLean (Harvard University, USA). Briefly, the plasmid myc (pCS2-MT) (Addgene) was digested with restriction endonucleases XbaI and XhoI, and asyn cDNA was inserted, resulting in the plasmids myc-WTasyn (pCS2-WTayn-MT) and myc-A30Pasyn (pCS2-A30P-MT). The plasmids Flag (pcDNA3-2XFLAG) and Flag-TRAF6 (pcDNA3-2XFLAG-Traf6) were a gift of Dr. Silvia Zucchelli (SISSA, Trieste, Italy).

Cell culture

SH-SY5Y neuroblastoma cells (CRL-2266, ATCC) were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The cells were transfected with the plasmids myc, myc-WTasyn, or myc A30Pasyn using FuGENE HD (Promega, Madison, WI, USA) by following the instructions of the manufacturer. Forty-eight hours after transfection, 400 μg/mL G418 (Invitrogen, Carlsbad, CA, USA) was added to the medium to select the transfected cells.

Immunoprecipitation

The SH-SY5Y cells were cotransfected with empty vector Flag or Flag-TRAF6 through FuGENE HD (Promega), following the manufacturer's instructions. The cells were lysed in lysis buffer (20 mM Na-Hepes, pH 7.7; 225 mM KCl, 1% Triton X-100) supplemented with an anti-protease cocktail (Roche, Basel, Switzerland). The cell lysates were incubated with an ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 4 °C. After washing, the immunoprecipitated proteins were eluted with 2x sodium dodecyl sulfate (SDS) sample buffer, boiled at 95 °C, and analyzed by Western blot.

Western blot

The concentration of the proteins was adjusted with sample buffer (0.125 M Tris-HCl; 4% SDS; 20% v/v glycerol; 0.2 M DTT; 0.02% bromophenol blue; pH 6.8). The proteins were loaded into 12% SDS-polyacrylamide gels for electrophoresis, transferred to nitrocellulose membranes, and stained with Ponceau S. The primary antibodies used were: anti-asyn 1:1000 (BD Healthcare, Franklin Lakes, NJ, USA) and anti-FLAG 1:500 (Sigma-Aldrich, St. Louis, MO, USA). HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit) were purchased from Sigma-Aldrich. The development of immunoblots was performed using ECL chemiluminescent substrate (Millipore, Billerica, MA, USA).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from SH-SY5Y cells were prepared as previously described (Kawamoto et al., 2008). Double-stranded oligonucleotides containing the NF-κB consensus sequence from Promega were end-labeled using T4 polynucleotide kinase (Promega) in the presence of γ-^{32}P dATP. Nuclear extracts (5 μg) were incubated with a ^{32}P-labeled NF-κB probe. The binding reaction was performed at room temperature for 30 min in reaction buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 20% glycerol, 0.25 μg/μL poly (dI-dC) and 2.5 mM dithiothreitol. DNA protein complexes were separated by electrophoresis through a 6% acrylamide:bis-acrylamide (37.5:1) gel in TBE (45 mM Tris, 45 mM Boric Acid, 0.5 mM EDTA) for 2 h at 150 V. Gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at -80 °C. For competition assays, 5 μg of nuclear extract was incubated with a specific competitor (unlabeled double-stranded NF-κB consensus oligonucleotide) or a nonspecific competitor (unlabeled transcription initiation factor IID [TFIID]). For supershift assays, antibodies against subunits of NF-κB (p50, p65, cRel, RelB 1:20) (Santa Cruz Biotechnology, CA, USA) were added into the binding reactions.

Measurement of TNF, IL-1β, and IL-10

The medium from SH-SY5Y cells was collected, and concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β and IL-10 were measured using commercial ELISA kits (R&D Systems), following the manufacturer's instructions. The detection limits of the method are 10.9 pg/mL (minimum) and 700 pg/mL (maximum).

Cell viability assay

Cell toxicity was observed by determining the LDH activity, measured by the CytoTox 96 detection kit (Promega). SH-SY5Y cells were seeded on 24-well plates (5×10^4 cell/well). As a positive control, cells were treated with 15 μL of lysis solution (0.9% Triton X-100) for 30 minutes. The fluorescence was measured with a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The LDH released was normalized to the total LDH released from the positive control.

Statistical analyses

Results were expressed as the mean ± SEM. The data were analyzed using the software GraphPad Prism. The statistical comparisons used for all the analyses were two-way ANOVA, followed by Tukey's test. P-values <0.05 were considered statistically significant.

Results

TRAF6 interacts with asyn

The interaction of TRAF6 with DJ-1, a protein also related to PD, has been shown by Zuchelli et al. (Zucchelli et al., 2010). Thus, we analyzed

whether TRAF6 could be associated with asyn. For this, we conducted coimmunoprecipitation experiments using SH-SY5Y cells transfected with Flag or Flag-TRAF6 with myc-WTasyn or the mutant form of asyn, myc-A30Pasyn. We demonstrate in Fig. 1 that TRAF6 can associate with WT or A30 P asyn.

Interaction between TRAF6 and asyn leads to increased NF-κB activity

TRAF6 is a major regulator of the activation of the NF-κB transcription factor. Likewise, asyn is known to increase the nuclear translocation of NF-κB and cause cell death (Prabhakaran et al., 2011). Thus, the interaction between TRAF6 and asyn could regulate this pathway. In Fig. 2A, we can observe the activation of NF-κB when cells were transfected with myc-WTasyn. There is a significant increase ($P < 0.001$) in NF-κB activity when the interaction between TRAF6 and WTasyn occurs compared to the group with only WTasyn. Surprisingly, the mutant form of asyn was not capable of activating the NF-κB signaling pathway with or without TRAF6 (Fig. 2A, B).

To determine which of the NF-κB subunits were translocated to the nuclei of the cells, we performed a supershift assay. The assay indicates the partial shift of subunit p50, while the antibody against subunit p65 was able to decrease the intensity of the formed complex (Fig. 2, lane 5, and 6). In a competition assay, represented in Fig. 2C, the upper complex was displaced by an excess of unlabeled NF-κB, but not by the TFIID double-stranded oligonucleotide consensus sequence, demonstrating the specificity of the NF-κB/DNA binding interaction. Thus, the heterodimer p65p50 of NF-κB is probably the most highly activated by TRAF6.

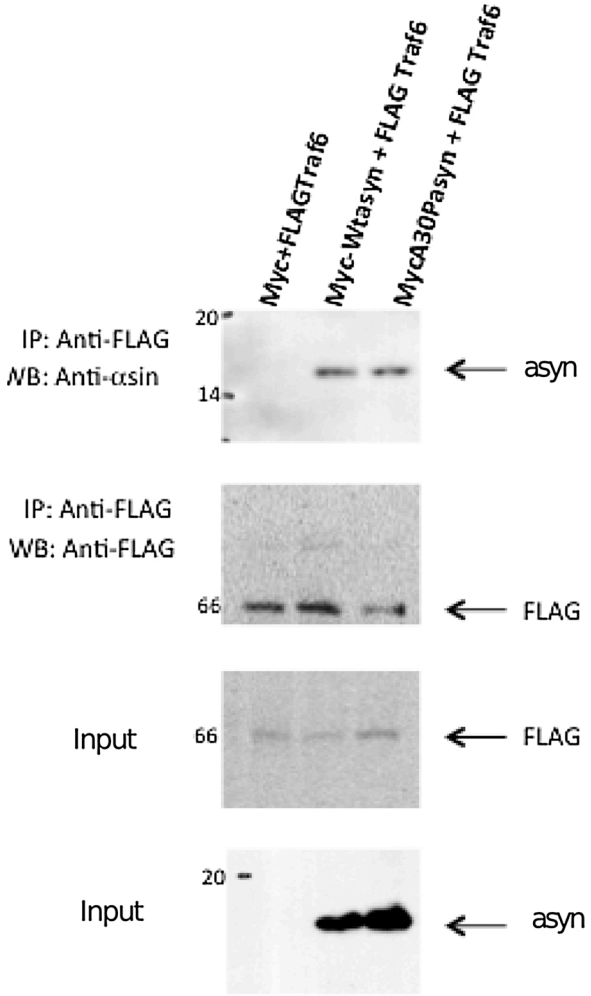

Fig. 1. *WTasyn and A30Pasyn bind to the ubiquitin ligase TRAF6.* SH-SY5Y cells were transfected with myc (empty vector), Myc-WTasyn or Myc-A30Pasyn and subsequently with FLAG-TRAF6. The protein asyn was developed with the antibody anti-asyn after immunoprecipitation with anti-FLAG antibody. As a control, the expression of FLAG or asyn protein was observed in the input (total lysis of the cells). The figure represents 3 independent experiments.

Interaction of TRAF-6 with asyn increases TNF and IL-1β and decreases IL-10 levels

The canonical NF-κB pathway induces the expression of various pro-inflammatory genes, including those encoding cytokines important to the innate and adaptive immune functions (Liu et al., 2017). A significant increase in both TNF and IL-1β and a decrease in IL-10 were observed in cells transfected with myc-WTasyn when TRAF6 was coexpressed compared with myc-WTasyn without TRAF6 (Fig. 3). Additionally, confirming the lack of activation of the NF-κB signaling pathway by the mutant form of asyn, no changes were observed between the myc-A30Pasyn groups (myc-A30Pasyn with or without TRAF6 coexpressed) as well as upon comparison to control groups (empty vector myc). These results indicate that despite the mutant form of asyn interacting with TRAF6, the pathways linked to TRAF6 are not activated.

WT asyn and TRAF6 cotransfected SH-SY5Y cells have decreased viability

The cells cotransfected with myc-WTasyn and Flag-TRAF6 present increased leakage of LDH compared to the group cotransfected with myc-WTasyn and Flag ($P < 0.05$) (Fig. 4). Additionally, the cells cotransfected with myc-WTasyn and Flag have lower levels of LDH detected in the medium compared to the control group (myc + flag) or A30Pasyn group (A30 P + Flag). Together, these results indicate that interaction of WT asyn with TRAF6 results in significantly lower cell viability.

Discussion

A main pathological feature of chronic PD is the presence of aggregates of proteins, known as LB (Spillantini et al., 1997). Several pieces of evidence imply the dysfunction of the ubiquitin-proteasome system in PD (Fiesel et al., 2014; Haj-Yahya et al., 2013; Wang et al., 2011).

The description of molecular components of LB and the discovery of new mediators that interact with these aggregates of proteins are important for the comprehension of the molecular mechanisms of PD and the identification of new therapeutic targets. The ubiquitin ligases are responsible for the formation of polyubiquitin chains in the proteins. They are frequently associated with neurodegenerative diseases since they are present in intracellular aggregates and participate in the formation of aggregates. For example, the ubiquitin ligases Parkin E3, the carboxyl terminus of Hsp70-interaction protein (CHIP), and seven in absentia homolog (SIAH) have been linked with LB (Chung et al., 2001; Liani et al., 2004; Shin et al., 2005).

The activity of TRAF6 has been characterized extensively in the context of activation of NF-κB (Chen, 2005; Chen, 2012). Interestingly, TRAF6 colocalizes in intracellular aggregates in the brains of PD patients, suggesting that the scavenging of TRAF6 can be a common mechanism in neurodegeneration. Moreover, structural studies observed that members of the TRAF and SIAH family have a highly similar c-terminal domain. Furthermore, Zucchelli and collaborators (Zucchelli et al., 2011) demonstrated the presence of TRAF6 in LB in the brains of postmortem PD patients.

In our study, we demonstrated that the ubiquitin ligase E3 TRAF6 interacts with asyn in a model of SH-SY5Y dopaminergic cells. To better elucidate the role of this interaction in signaling that involves the NF-κB pathway, we analyzed the activation of this transcription factor when it interacts with TRAF6.

As previously reported, asyn can activate the NF-κB pathway (Yuan

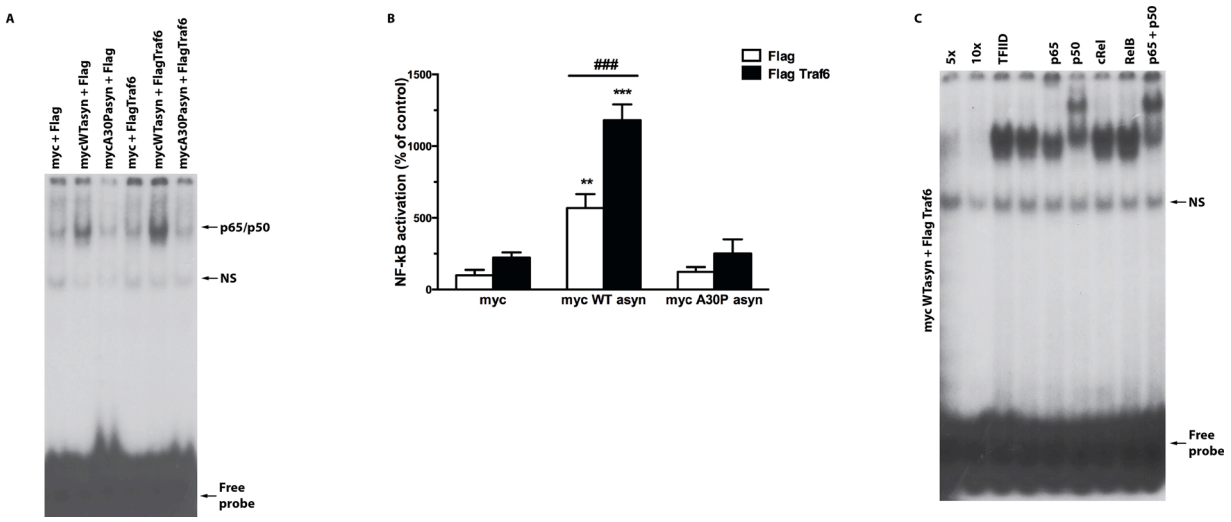

Fig. 2. *Influence of TRAF6 interaction with asyn in NF-κB activation.* **A.** Representative EMSA autoradiography. The NFκB-specific band (p65/p50 heterodimers) is indicated by an arrow. NS represents nonspecific binding. **B.** Densitometric analysis of p65/p50 heterodimers of the nuclear extracts of SH-SY5Y cells. The data represent the mean ± SEM of 3 independent experiments. Two-way ANOVA followed by Tukey's test: $^{**}P < 0.01$ and $^{***}P < 0.001$ versus cells cotransfected with myc and Flag; $^{###}P < 0.001$ cells cotransfected with mycWTasyn and Flag versus cells cotransfected with mycWTasyn and Flag-TRAF6. Control group = SY-SY5Y cells cotransfected with myc and Flag. **C.** Supershift and competition assay were performed on nuclear extract of SH-SY5Y cells cotransfected with mycWTasyn and Flag-TRAF6. First and second lanes (from left to right) represent the presence of unlabeled specific oligonucleotides (NFκB consensus sequence, 5-fold and 10-fold molar excess, respectively). Lane 3 represents the presence of nonspecific oligonucleotides (TFIID consensus sequence at 10-fold molar excess). Supershift assay was performed in absence or presence of antibodies against NF-κB subunits p65, p50, cRel, RelB and p65 + p50, as indicated.

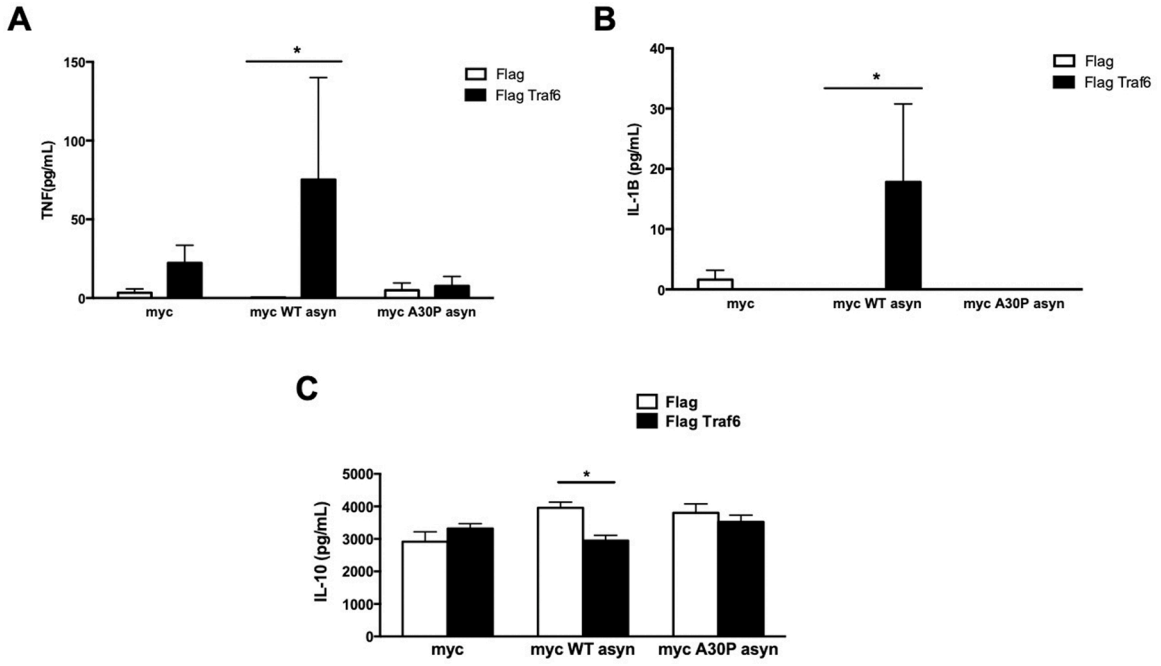

Fig. 3. *Influence of TRAF6 and asyn interaction on TNF, IL-1β and IL-10 levels.* Supernatants of the transfected cells with myc, myc-WTasyn or myc-A30Pasyn and subsequently with FLAG or FLAG-TRAF6 were collected and used to measure TNF, IL-1β and IL-10 levels by ELISA (n = 6). **A.** Increase in TNF release in the myc WT asyn + FlagTRAF6 group compared to myc WT asyn + Flag, $^{*}P < 0.05$. **B.** Increase in IL-1β release in the myc WT asyn + FlagTRAF6 group compared to myc WT asyn + Flag. **C.** Decrease in IL-10 release in the myc WT asyn + FlagTRAF6 group. The data represent the mean ± SEM of 6 independent experiments.

et al., 2008a). We observed that the cells transfected with WT asyn showed increased NF-κB activity when compared to the control group (cells transfected with control plasmids). Interestingly, the interaction of WT asyn with TRAF6 significantly increases this activation and leads to augmented cell death when compared to its control, pinpointing that the interaction of TRAF6 with asyn can lead to harmful effects in SH-SY5Y cells. Since TRAF6 is expressed in LB of PD patients, where it can interact with WT asyn (Zucchelli et al., 2010), the present data indicate that the expression and interaction of TRAF6 with WT asyn in SH-SY5Y cells show a phenotype similar to that observed in PD patients, thus validating this model to study the influence of this pathway in PD.

Additionally, the role of NF-κB in regulating pro-and anti-inflammatory genes and its influence in neurodegenerative diseases are well known (Camandola and Mattson, 2007). Therefore, the increased activation of NF-κB shown after the interaction of TRAF6 with asyn could be the cause of increased cell death. The overexpression of asyn

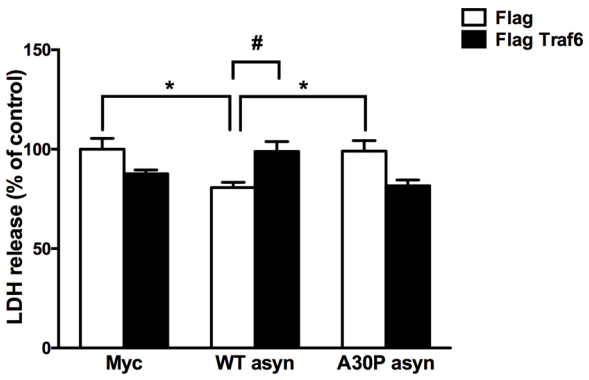

Fig. 4. *Increase of LDH liberation from SH-SY5Y cells cotransfected with myc-A30Pasyn and Flag-TRAF6.* SH-SY5Y cells were transfected with myc, myc-WTasyn or myc-A30Pasyn and subsequently with FLAG or FLAG-TRAF6. LDH assay was performed and read at absorbance 490 nm. The data represent the mean ± SEM of 5 independent experiments. Two-way ANOVA followed by Tukey's test: *P < 0.05 cells cotransfected with myc-WTasyn and Flag versus cells cotransfected with myc and Flag and cells cotransfected with myc-A30Pasyn and Flag; #P < 0.05 cells cotransfected with myc-WTasyn and Flag-TRAF6 versus cells cotransfected with myc-WTasyn and Flag. Control group = SH-SY5Y cells cotransfected with myc and Flag.

downregulated the anti-apoptotic Bcl-2 expression and upregulated the pro-apoptotic glycogen synthase kinase 3β (GSK3β) protein level in SH-SY5Y cells, suggesting that the proapoptotic effects of asyn might be mediated at least in part by the NF-κB signaling pathway, which involves GSK3β (Yuan et al., 2008b). Our data showed that the interaction of TRAF6 and WT asyn in SH-SY5Y cells increased the levels of the proinflammatory cytokines TNF and IL-1β and decreased the levels of IL-10, an anti-inflammatory cytokine that balances the immune response in the CNS and prevents deleterious inflammation in the brain (Lobo-Silva et al., 2016). Therefore, the interaction of WT asyn with TRAF6 significantly increases the proinflammatory status that might be linked to the increase in cell death.

Surprisingly, the mutant form A30 P asyn did not experience increased cell death or higher activity of NF-κB and changes in cytokines when compared to its control. This may be due to a lower propensity of the A30 P mutant to form aggregates of asyn, unlike other mutants such as E46 K and G51D (Lazaro et al., 2014). It is also important to consider that A30 P asyn has a disrupted N-terminus that greatly impairs its binding to phospholipids and disrupts the α-helical conformation (Bridi and Hirth, 2018).

Even though there is binding between A30 P asyn and TRAF6, another possibility to explain the differences between mutant and WT asyn is that the function of asyn could be impaired or the binding of A30 P asyn to TRAF6 does not promote the same conformational changes in TRAF6 that occur with WT asyn interaction. In addition, we cannot rule out the involvement of other transcription factors (such as AP-1) in the signaling cascade activated by TRAF6 as being responsible for the differences observed between WT asyn and A30 P asyn in SH-SY5Y. Future investigation will be necessary to elucidate our new report showing the lack of activation of NF-κB when mutant asyn was coexpressed with TRAF6 in the cells.

Conclusion

These data indicate that the interaction of TRAF6 with asyn is detrimental to the cell, leading to upregulation of the NF-κB signaling pathway and cell death. Interestingly, the A30 P asyn mutation interacted with TRAF6 but was not able to activate NF-κB. Considering that the inflammatory response is adaptive, it is possible that, in our model, activation of NF-κB induced by myc-WTasyn when TRAF6 is coexpressed produced a persistent inflammatory reaction leading to cell death. To corroborate this, further experiments are needed to investigate the role of asyn with TRAF6 in our cell model as well as in vivo studies.

Conflicts of interest

The authors report no declarations of interest.

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Authors' contributions

LMY and CS designed the study and wrote the paper. AM and MG performed and analyzed the immunoprecipitation experiments. ADS designed and constructed the plasmids myc-WTasyn and myc-A30Pasyn. PFK helped with the experiments and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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