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Nitrosative stress-induced Parkinsonian Lewy-like aggregates prevented through polyphenolic phytochemical analog intervention

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ABSTRACT

Nitrosative stress has recently been demonstrated as a causal in a select sporadic variant of Parkinson's (PD) and Alzheimer's (AD) diseases. Specifically, elevated levels of NO disrupt the redox activity of protein-disulfide isomerase, a key endoplasmic reticulum-resident chaperone by *S*-nitroso modification of its redox-active cysteines. This leads to accumulation of misfolded AD- and PD-specific protein debris. We have recently demonstrated *in vitro* that polyphenolic phytochemicals, curcumin and masoprocol, can rescue *S*-nitroso-PDI formation by scavenging NO*x*. In this study, using dopaminergic SHSY-5Y cells, we have monitored the aggregation of green-fluorescent protein (GFP)-tagged synphilin-1 (a known constituent of PD Lewy neurites) as a function of rotenone-induced nitrosative stress. Importantly, we demonstrate a marked decrease in synphilin-1 aggregation when the cell line is previously incubated with 3,5-bis(2-flurobenzylidene) piperidin-4-one (EF-24), a curcumin analogue, prior to rotenone insult. Furthermore, our data also reveal that rotenone attenuates PDI expression in the same cell line, a phenomenon that can be mitigated through EF-24 intervention. Together, these results suggest that EF-24 can exert neuroprotective effects by ameliorating nitrosative stress-linked damage to PDI and the associated onset of PD and AD. Essentially, EF-24 can serve as a scaffold for the design and development of PD and AD specific prophylactics.

1. Introduction

A hallmark event characteristic of neurogenerative disorders such as Lewy body dementia, Alzheimer's (AD) and Parkinson's diseases (PD) is the accumulation of aggregated proteins to often form Lewy-bodies in the cytosol of human neuronal cells [1–11]. This commonality exists in spite of the fact that causals for AD and PD are different, depend upon whether familial or sporadic, and differ in the proteins constituting the aggregates and Lewy neurites. There is also debate whether specific protein aggregates representative of these diseases are truly the cause of the disease or only a mechanism to save the cell upon onset of the neuronal pathology [12].

Among a plethora of known intra- and extracellular factors associated with the etiology of AD and PD including mitochondrial dysfunction, inflammation, diet, etc., recent studies have demonstrated that in a particular sporadic form of AD and PD, a key endoplasmic reticulum-resident oxidoreductase chaperone becomes chemically modified because of high levels of nitrosative stress [13–19]. A common feature observed in the neuronal cells of AD and PD victims in this sporadic variant was the attachment of nitric oxide (NO) to the redox-active cysteines of protein-disulfide isomerase (PDI) to form *S*-nitroso-PDI (Fig. 1A) [5,7]. The formation of *S*-nitroso-PDI coupled with the pathogenesis of AD and PD making the oxidoreductase a chief target for the prevention of these two neurodegenerative disorders in the nitrosative-stress-linked variant of the diseases [5,7].

Our laboratory recently demonstrated that the polyphenolic phytochemicals curcumin and masoprocol can scavenge NO*x* species from model NOx donors [20]. Additionally, these polyphenols have been shown to intervene in a variety of disease processes [21–26]. Furthermore, we have also demonstrated *in vitro* that they can rescue PDI from becoming chemically modified under nitrosative stress [20]. This was possible by following the oxidative regeneration of bovine pancreatic ribonuclease A (RNase A), a PDI substrate, under control and nitrosative stress conditions [20]. Our results indicated that both curcumin and masoprocol can restore PDI activity to native levels even in the presence of NO*x* sources. Mass spectrometric results revealed that both polyphenols scavenge NO*x* to form stable adducts [20]. In addition, they were

Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease; PDI, protein disulfide isomerase; EF-24, 3,5-bis(2-flurobenzylidene) piperidin-4-one.

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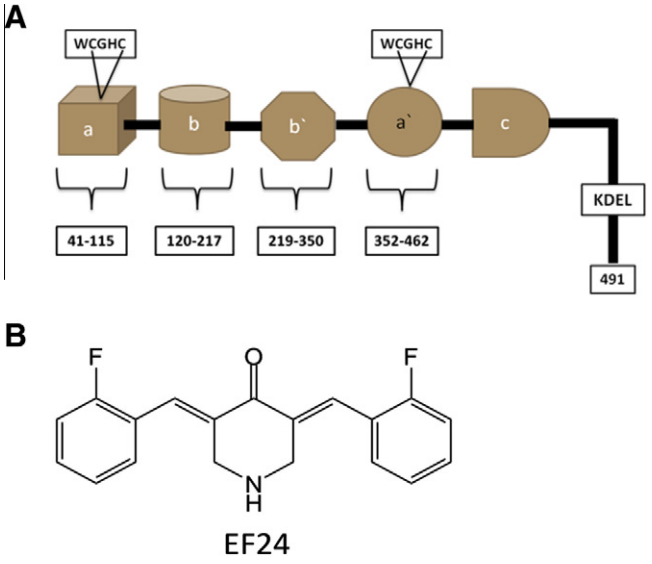

Fig. 1. (A) Schematic representation of protein-disulfide isomerase (PDI) [32]; (B) EF-24 [27].

found to be capable of scavenging hydroxyl radicals generated using Fenton chemistry [manuscript in preparation].

In this report we have overexpressed green-fluorescent protein-tagged synphilin-1 in SHSY-5Y cells and monitored its aggregation as a biomarker for PD. Our results reveal that exposure of this cell line to rotenone, a mitochondrial reactive oxygen species elevator, leads to the aggregation of synphilin-1 as observed by fluorescence microscopy and consistent with previous reports that NO influences Lewy body formation via PDI modification [7]. Importantly, cells that were preincubated with 3,5-bis(2-flurobenzylidene) piperidin-4-one (EF-24), a bioavailable curcumin analog, prior to rotenone insult demonstrated a marked resilience to synphilin-1 aggregation (Fig. 1B) [27]. These results suggest that it may be possible to mitigate nitrosative-stress induced aggregates in cell lines using bioavailable polyphenolic phyto-analogs. Furthermore, it opens avenues for the design and development of more effective and less toxic analogs prophylactics against nitrosative-stress linked AD and PD.

2. Materials and methods

2.1. Reagent and cell line

EF-24 was synthesized in collaboration with Dr. Katja Michael (Dept. of Chemistry, UTEP) using a previously reported procedure [27]. Further purification was performed using reversed-phase HPLC (Supelco, C18 column; 0–100% acetonitrile/100 min) to collect the principal peak. Characterization of the principal peak by mass spectrometry revealed a molecular weight in good agreement with the expected mass of EF-24 [27]. HPLC analysis of re-purified EF-24 showed purity $\geqslant$95% by peak area analysis. Tetranitromethane (TNM) and rotenone were obtained from Sigma–Aldrich (St. Louis, MO). Other reagents were purchased as follows: Mouse monoclonal to PDI (Abcam, Cambridge, MA), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, Cell Signaling, Danvers, MA), horseradish peroxidase (HRP)-conjugated goat anti-mouse (KPL Biomedical) and the neuroblastoma cell line SHSY-5Y (from ATCC, Manassas, VA).

2.2. Characterization of NOx adducts of EF-24

A stock solution of EF-24 (prepared by weight in acetonitrile) was diluted into a buffer (pH 8, 100 mM Tris–HCl) to obtain concentrations ranging from 10 to 200 μM. Tetranitromethane was added from a stock solution (freshly prepared by weight in acetonitrile) to EF-24 so as to obtain different ratios of TNM/EF-24. The samples were separated and analyzed using reversed-phase HPLC (Supelco C18 column; 1% acetonitrile/min). Collected peaks were lyophilized and analyzed on a Q-TOF mass spectrometer (BBRC, UTEP).

2.3. Western blot analysis of PDI

Dopaminergic SHSY-5Y transfected cells were subjected to different treatments, followed by washing the cells with cold Tris-buffered saline, collected by centrifugation (3003 $\times$ *g*, 5 min at 48 °C), and lysed by sonication in a buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 2% (w/v) SDS and protease inhibitors. Total protein concentration was measured using a bicinchonic acid kit and BSA as a standard (Pierce Biotechnology Inc., Rockford, IL). Equal volumes of protein (approximately 10 μg per lane) were subject to SDS–polyacrylamide gel electrophoresis and then transferred to polyvinyldinefluoride (PVDF) membranes. Blots were incubated in blocking buffer (5%, w/v, dried skimmed milk in Tris-buffered saline, pH 7.4, and 0.1% Tween 20) followed by incubation with anti-PDI monoclonal antibody (1:500) or anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, 1:1000) for 1 h and then with horseradish peroxidase (HRP)-conjugated goat anti-mouse (KPL Biomedical) for 30 min. The signal was visualized by chemiluminescence (ECL-plus or SuperSignal West Pico Chemiluminescent Substrate) according to the manufacturer's instructions (Amersham or Pierce Biotechnology, Inc.).

2.4. Preparation of EGFP-synphilin-1 fusion protein

The full-length cDNA of the human synphilin-1 (pENTR 221 from Invitrogen; Genbank accession code NP 005451) was amplified via PCR using the primer set containing the restriction sites for EcoRI (forward) and BamHI (reverse). After digestion with the corresponding enzymes, the PCR product was cloned into the pEGFP-C2 expression vector (Clontech. Palo Alto, CA). The sequence of synphilin-1 was verified by automated DNA sequencing reaction. The synphilin-1/pEGFP-C2 construct was utilized to transiently transfect neuroblastoma cells.

2.5. Cell culture and treatment

SHSY-5Y (human neuroblastoma) cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin. The cells were grown at 37 °C with 5% carbon dioxide. SHSY-5Y cells (1×10^6 cells/well) were seeded onto glass coverslips in 6-well plates and incubated for 12–16 h. Cell transfections were performed the following day with pEGFP-C2 control or the fusion protein GFP-*synphilin*-1, as recommended by manufacturer using effectene reagent (Qiagen, Valencia, CA). After transfection, the cells were incubated overnight to allow expression of proteins. Cells were treated with vehicle alone (DMSO) or with 1 μM EF-24 for 6 h followed by exposure to 300 nM of rotenone for 12 h. After incubation, cells were prepared for microscopy as described below.

2.6. Confocal microscopy and immunocytochemistry

Cells transfected with vector or EGFP-synphilin-1 were washed after treatment, fixed with 4% paraformaldehyde in PBS, stained with DAPI and mounted under ProLong antifade medium (Molecular Probes). To stain for PDI, cells were fixed as above, permeabilized with 0.1% (w/v) saponin in PBS, blocked with PBS plus 5% goat serum, 5% FBS and 0.1% TWEEN 20, followed by incubation with