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Interplay of salicylaldehyde, lysine, and M^{2+} ions on α-synuclein aggregation: Cancellation of aggregation effects and determination of salicylaldehyde neurotoxicity

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ABSTRACT

In this study, α-synuclein was treated *in vitro* with salicylaldehyde (SA), lysine (lys) and M^{n+} (Cu^{2+} or Zn^{2+}) in various ratios. SA induced aggregation of α-syn in the ratio of 1:500 (α-syn:SA) after incubation (pH 7.4, PBS buffer, 16–24 h). Free lys can thus scavenge SA, inhibiting the aggregation of α-syn up to ~63% (α-syn:SA:lys = 1:1000:5000). When Cu^{2+} and Zn^{2+} are added to SA and α-syn, protein aggregation is induced. In the case of Zn^{2+}, the aggregation of α-syn increased to 74% (ratio = 1:1000:50). Fluorescence studies support the production of protein-bound Zn^{2+}-salicylaldimine species. For Cu^{2+}, aggregation of α-syn was shown (138%). Thus, possible protective or inducing effects of lys, Cu^{2+} and Zn^{2+} may exist with α-syn. α-Syn, SA and Cu^{2+} can undergo complexation (fluorescence, CD and MALDI data). Cellular toxicity of SA (700 μM), Zn^{2+} (700 μM) and Cu^{2+} (700 μM) on SH-SY5Y (1×10^5 cells) showed 9.8%, 38.0% and 14.4% compared to control values. Combinations showed more severe toxicities: 71.9% and 93.1% for SA (70 μM) + Cu^{2+} (700 μM) and SA (70 μM) + Zn^{2+} (700 μM), respectively, suggesting complexation itself may be toxic.

1. Introduction

Molecular neurodegeneration continues to be rigorously researched in the context of possibly discovering blockbuster drugs for Alzheimer's disease (AD) and Parkinson's disease (PD). There is still a general lack of understanding of the particulars of disease etiology, which, when combined with the prevalence, seriousness, complexity, and incurability of these disorders, underscores the importance of both undertaking new investigations as well as scrutinizing existing research directions (Bush, 2003; Jeffrey, 2009; Uversky, 2007). On the molecular level, protein misfolding is thought to be a root cause in such neuronal pathology. Misfolding can lead to facile aggregation and can stem from protein oxidation or other changes. Metal binding, among other factors, can induce protein misfolding, which can, in turn, give aggregation. There are a variety of proteins implicated in such misfolding: β-amyloid, α-synuclein (α-syn), superoxide dismutase (SOD), etc. α-Syn is considered the hallmark protein of PD as it is found in excess as deposits (Lewy bodies). In the brain tissue of PD patients, filaments of aggregated α-syn accumulate in dopaminergic neurons (Spillantini et al., 1998). α-Syn is an unstructured protein composed of 140 amino acid residues, and can associate easily with biological membranes (Davidson et al., 1998). It is found in high concentration in pre-synaptic nerve terminals (Davidson et al., 1998; McLean et al., 2000). α-Syn can be discussed as having three major domains: (*i*) An α-helical *N*-terminal region of six repeated amino acids (namely, KTKEGV), similar to the lipid binding domains of apolipoproteins. (*ii*) The middle region possesses hydrophobic residues and can organize partly into a β-pleated sheet. (*iii*) The C-terminal region is rich in glutamate residues and is very acidic (Uversky, 2007).

There are many possible post-translational changes, and possible for proteins such as α-syn. For example, phosphorylation, nitration, oxidation, ubiquitination, can occur (Beyer, 2006), which impart phosphate, nitro, oxygen groups, and ubiquitin protein, respectively. Certain such changes appear to relate to the

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MDVFMKGLSK[10] AKEGVVAAAE[20] KTKQGVAEAA[30]

GKTKEGVLYV[40] GSKTKEGVVH[50] GVATVAEKTK[60]

EQVTNVGGAV[70] VTGVTAVAQK[80] TVEGAGSIAA[90]

ATGFVKKDQL[100] GKNEEGAPQE[110] GILEDMPVDP[120]

DNEAYEMPSE[130] EGYQDYEPEA[140]

Fig. 1. Primary sequence of α-syn from the *N*- to C-termini; Lys (K) residues are highlighted. Residues 1–60: amphipathic region (red); 61–95: hydrophobic region (black); 96–140: proline-rich acidic region (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

neurological diseased state. Serine and threonine residues (esp. Ser 129) can become easily phosphorylated and enhance the formation of cytoplasmic inclusion bodies (Smith et al., 2005). Nitration is related to di-tyrosine crosslinking (Souza et al., 2000). Ubiquitination of α-syn is thought to be associated with Lewy body formation (Tofaris et al., 2003). Nitration and oxidation are usually related to oligomer formation arising from covalent linkages between monomers. Combinations of imine formation and metal ion behavior have not been matched before, to the best of our knowledge, with investigations of α-syn.

Oxidation of biomolecules can produce many kinds of toxic aldehydes. Dopamine can be transformed into 3,4-dihydroxyphenylacetaldehyde (DOPAL) by the enzyme monoamine oxidase in the membranes of mitochondria (Burke et al., 1999, 2003) bestowing it with both a catechol and aldehyde functionality. Catechol can be oxidized into cysteine-reactive quinone; aldehydes are reactive to lys residues and can generate Schiff bases (Rees et al., 2009). α-Syn has 15 lys residues, 11 in the *N*-terminal region, one in the middle region, and three in the C-terminal region (Fig. 1). DOPAL can trigger initial α-syn aggregation which may lead to toxic protein oligomers in, e.g., SH-SY5Y cell cultures (Burke et al., 2008).

It is known that aldehydes, some of which are products of lipid peroxidation, induce α-syn aggregation. These include 4-hydroxynonenal (HNE), 4-hydroxy-2-hexenal (HHE), 4-oxo-2-nonenal (ONE), and acrolein (Long et al., 2008; Nasstrom et al., 2009; Qin et al., 2007; Shamoto-Nagai et al., 2007). HNE is a product of lipid peroxidation and can bind to α-syn via Michael addition and can subsequently induce aggregation (Qin et al., 2007). HNE and malondialdehyde (MDA) can inhibit aldehyde biotransformation that can induce the increasing levels of DOPAL (Jinsmaa et al., 2009). HHE is toxic to primary cultures of cerebral cortical neurons. Thiol scavengers can decrease the toxicity of HHE (Long et al., 2008). ONE has been found to completely convert α-syn monomers into oligomeric forms, but not into fibrillar forms. The oligomer form (rich with β-sheets) is very stable against, e.g., sodium dodecyl sulfate degradation (Nasstrom et al., 2009). Acrolein is also a product of lipid peroxidation; it can allow α-syn to aggregate into oligomers. The oligomer form of α-syn is accumulated in dopaminergic neurons and capable of inhibiting proteasomal activity (Shamoto-Nagai et al., 2007). Further, cholesterol can be oxidized *in vivo* to the aldehyde (Bieschke et al., 2006). Cholesterol oxidation products are elevated in Lewy body dementia brain tissue (Bieschke et al., 2006; Bosco et al., 2006). Cholesterol aldehyde can induce $A\beta^{(1-40)}$ aggregation via covalent modification. However, in the case of α-syn, aggregation can occur *without* formation of a covalent protein linkage. Separately, certain molecules have been studied as aldehyde scavengers. These include hydralazine, methoxyamine, and aminoguanidine (Nilsson, 1999; Sasaki et al., 2009), previously studied with $A\beta^{(1-40)}$ (Bieschke et al., 2006).

Some studies relating to metal ions and α-syn exist in the literature (Kowalik-Jankowska et al., 2005, 2006; Lucas and Lee, 2009; Peng et al., 2010). Some transition metal ions such as Cu^{2+}, Fe^{2+}, and Zn^{2+} can catalyze oxidation of α-syn to give protofibrils and oligomers. They usually combine with hydrogen peroxide and other reactive oxygen species (ROS) to induce covalent modifications and oligomerization. In the presence of reducing agent, e.g., dithiothreitol (DTT), $Fe^{2+/3+}$, and hydrogen peroxide, forms of α-syn that are curved and branched can be produced (Cole et al., 2005).

SA, while a natural species, has not been previously investigated in the context of neurons, animal brains, or the human body to the best of our knowledge. SA is a constituent of buckwheat groats (Janeš and Kreft, 2007) and *Filipendula vulgaris* (Radulovic et al., 2007). Further, it shows remarkable inhibition effects on the growth of certain species of bacteria and fungi (Radulovic et al., 2007), and serves to repel insects from particular flowers (Koschier et al., 2007); it is also known to induce dermatitis (Aalto-Korte et al., 2005). Importantly, it has not been suspected in having a role in molecular neurodegenerative disorders. Instead, SA could be considered a possible fluorogenic model for pyridoxal, a biological version of SA. SA and its moieties are common building units in fluorophores and metal chelation in biological and inorganic systems (Khatua et al., 2009a,b; Prudent et al., 2008); pyridoxal yields very limited fluorescence properties. Thus, herein we pursue with academic interest the notion whether there is any toxicity with SA, even though it is not formed endogenously and not suspected as a factor in neurodegenerative diseases. We undertook protein and cell studies with SA, but needed to do so at relatively high concentrations to effect the intended aggregation effects. All in all, this contribution might (i) lead to new ways of quantifying the number of lys residues in proteins, (ii) help prepare fluorescent versions of proteins, or (iii) perhaps help quantify aspects of protein folding. In the last part of this paper, we went further and directly studied *in vitro* neuronal cellular toxicity studies of SA alone and in conjunction with metal ions.

According to Lipinski's rules, prospective orally active drug molecules are likely to have certain characteristics. Specifically, they are likely to not have hydrogen bond donors of more than five in number, hydrogen bond acceptors of more than ten in number, molecular weights of more than 500 Da, and calculated log *P* values of greater than five, and have poor solubility and permeability in body systems (Lipinski et al., 2001). Generally, compounds which have low molecular weight (less than 400–600 Da) and good lipid (fat) solubility can diffuse across the Blood Brain Barrier (BBB) (Banks, 2009; Pardridge, 1995, 1998). SA shows good solubility in organic solvents, and has a low molecular weight, 122.12 Da. From this information, it is guestimated that SA might penetrate the BBB and could conceivably influence, or be utilized in applications of, neurodegenerative and brain diseases.

Herein, we shall study the effect of SA on α-syn via biochemical methods. The interplay of this aldehyde in pure aqueous media with subsequent presence of Zn^{2+} and Cu^{2+}, and lys, described below is interesting at least in an academic sense, and plays important roles via self assembly in the context of protein aggregation tendencies.

2. Experimental methods

2.1. Effecting α-syn aggregation with SA, L-lysine, Zn^{2+} and Cu^{2+}

2.1.1. Materials and methods

Human wild-type α-syn recombinant from *Escherichia coli*, was purchased from ProSpec-Tany TechnoGene Ltd. The purity was greater than 95% by RP-HPLC, according to the literature provided