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Low molar excess of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal promote oligomerization of alpha-synuclein through different pathways

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ABSTRACT

Aggregated alpha-synuclein is the main component of Lewy bodies, intraneuronal inclusions found in brains with Parkinson's disease and dementia with Lewy bodies. A body of evidence implicates oxidative stress in the pathogenesis of these diseases. For example, a large excess (30:1, aldehyde:protein) of the lipid peroxidation end products 4-oxo-2-nonenal (ONE) or 4-hydroxy-2-nonenal (HNE) can induce alpha-synuclein oligomer formation. The objective of the study was to investigate the effect of these reactive aldehydes on alpha-synuclein at a lower molar excess (3:1) at both physiological (7.4) and acidic (5.4) pH. As observed by size-exclusion chromatography, ONE rapidly induced the formation of alpha-synuclein oligomers at both pH values, but the effect was less pronounced under the acidic condition. In contrast, only a small proportion of alpha-synuclein oligomers were formed with low excess HNE-treatment at physiological pH and no oligomers at all under the acidic condition. With prolonged incubation times (up to 96 h), more alpha-synuclein was oligomerized at physiological pH for both ONE and HNE. As determined by Western blot, ONE-oligomers were more SDS-stable and to a higher-degree cross-linked as compared to the HNE-induced oligomers. However, as shown by their greater sensitivity to proteinase K treatment, ONE-oligomers, exhibited a less compact structure than HNE-oligomers. As indicated by mass spectrometry, ONE modified most Lys residues, whereas HNE primarily modified the His50 residue and fewer Lys residues, albeit to a higher degree than ONE. Taken together, our data show that the aldehydes ONE and HNE can modify alpha-synuclein and induce oligomerization, even at low molar excess, but to a higher degree at physiological pH and seemingly through different pathways.

1. Introduction

Intraneuronal inclusions of aggregated alpha-synuclein, known as Lewy bodies, are the neuropathological hallmark of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [1]. The physiological function of alpha-synuclein has not been completely elucidated, but the native protein has been suggested to be involved in neurotransmitter release [2], modulation of synaptic plasticity [3] and vesicle recycling [4]. Furthermore, recent studies indicate that alpha-synuclein might have an important role in the assembly process of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, by binding to the SNARE protein VAMP2 [5,6].

Alpha-synuclein consists of 140 amino acid residues and can be divided into three structurally distinct domains: a lipid-binding amphipathic N-terminal region (1–60), a central hydrophobic region (residues 61–95) and a negatively charged C-terminal region (residues 96–140). During the aggregation process, natively unstructured monomeric alpha-synuclein undergo conformational changes into more folded intermediate molecular species with increasing molecular weight, such as oligomers and protofibrils [7]. Continued

Abbreviations: PD, Parkinson's disease; DLB, dementia with Lewy bodies; ONE, 4-oxo-2-nonenal; HNE, 4-hydroxy-2-nonenal; SEC, size-exclusion chromatography; AFM, atomic force microscopy; PK, proteinase K; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay

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polymerization ultimately gives rise to insoluble β-sheet rich fibrils [8,9]. The formed oligomers can either be on-pathway, i.e. on their way of forming fibrils, or off-pathway, i.e. adopting a kinetically stable conformational state that precludes them from polymerizing into fibrils [10]. The underlying mechanisms by which aggregated forms of alpha-synuclein cause neurodegeneration are not completely understood, but growing evidence suggests that oligomers display particularly neurotoxic properties [11–14].

Six point mutations (A30P, E46K, H50Q, G51D, A53E and A53T) have been found to be associated to early onset familial forms of PD and DLB [15–20]. Furthermore, patients carrying duplications and triplications of the alpha-synuclein gene develop familial forms of the diseases [21,22], indicating a crucial role of alpha-synuclein aggregation in the pathogenesis of PD and DLB.

Oxidative stress appears to play a central role in the pathogenesis of neurodegenerative disorders. The brain has a high consumption of oxygen in combination with a low level of antioxidant scavenging enzymes [23]. Moreover, a high amount of polyunsaturated fatty acids (PUFAs), and high levels of redox transition metals lead to an excess production of reactive oxygen species (ROS) [24–26]. As ROS are highly unstable, they can initiate lipid peroxidation of PUFAs, which is a central event of free radical mediated injury [26]. In this process, a range of secondary products are generated, such as the reactive aldehydes 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) [27–29]. Both ONE and HNE can form covalent adducts with proteins, thereby potentially altering their structure and thus impairing protein function [30]. The chemical structures of ONE and HNE are nearly identical and they only differ at their C4 position, where ONE has a carbonyl group and HNE has a hydroxyl group (Fig. 1A) [30–32].

It has been shown that HNE-modified proteins can be observed to a higher extent in nigral neurons in both PD and DLB [33,34]. Moreover, it has been demonstrated that both ONE and HNE readily react with alpha-synuclein and induce aggregation in vitro [35–40]. Alpha-synuclein can be modified by ONE and HNE via Michael addition, which generates adducts through side chains of Cys, His or Lys residues [41,42]. Moreover, ONE and HNE also modify proteins through Schiff base formation with Lys residues [41,42]. However, the chemistry of ONE is much more diverse, e.g., the Schiff base over time can give rise to a 4-ketoamide which is isomeric to a Lys-ONE Michael adduct [43]. Alpha-synuclein contains no Cys residues, one His (at position 50) and fifteen Lys residues (located between amino acids 6 and 102) (Fig. 1B). Our group has recently demonstrated that ONE and HNE-induced alpha-synuclein oligomers differ in morphological and biochemical characteristics [40]. However, in these previous studies a high molar excess (20:1 – 30:1) of ONE and HNE were used [35–40]. Here, we wanted to investigate the effect of the aldehydes on alpha-synuclein oligomerization at a lower, and probably more physiological, molar excess (3:1). Moreover, as acidic pH has been shown to increase the aggregation rate for unmodified alpha-synuclein [7,44], we also wanted to investigate the effect of the aldehydes on oligomerization at an acidic pH.

2. Material and methods

2.1. Chemicals

ONE (5 mg/ml) in 99% methyl acetate and HNE (10 mg/ml) was supplied in 99% ethanol, (Cayman Chemical Ann Arbor, MI). Recombinant alpha-synuclein was expressed and purified as previously described [36]. Acetonitrile (ACN), acetic acid (HAc), trifluoroacetic acid (TFA) and ammonium bicarbonate (AmBi) were obtained from Merck (Darmstadt, Germany). Trypsin and Glu-C (sequencing grade from bovine pancreas and Staphylococcus aureus, respectively; Roche diagnostic, Basel, Switzerland) was used for digestion of each alpha-synuclein sample.

2.2. Protein determination

The alpha-synuclein concentration was determined using Pierce BCA kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions.

2.3. Modification of alpha-synuclein with ONE or HNE

Recombinant alpha-synuclein (140 μM) was buffer exchanged to a 50 mM sodium phosphate buffer, pH 5.4 or pH 7.4, using Zeba™ desalt spin columns (Thermo Scientific). To generate aldehyde modified alpha-synuclein species, ONE or HNE was added to alpha-synuclein (140 μM), with a final concentration equivalent of a molar ratio of 3:1 (ONE/HNE: alpha-synuclein). Then, samples were quiescently incubated at 37 °C for six different time points: 15 min, 30 min, 45 min, 1 h, 8 h and 24 h. In a separate experiment, samples were incubated for 96 h.

2.4. Production of unmodified alpha-synuclein oligomers through lyophilization

The unmodified oligomers were prepared according to previous protocols [45,46]. First, 600 μL of recombinant alpha-synuclein at a concentration of in MilliQ water was lyophilized using a VirTis

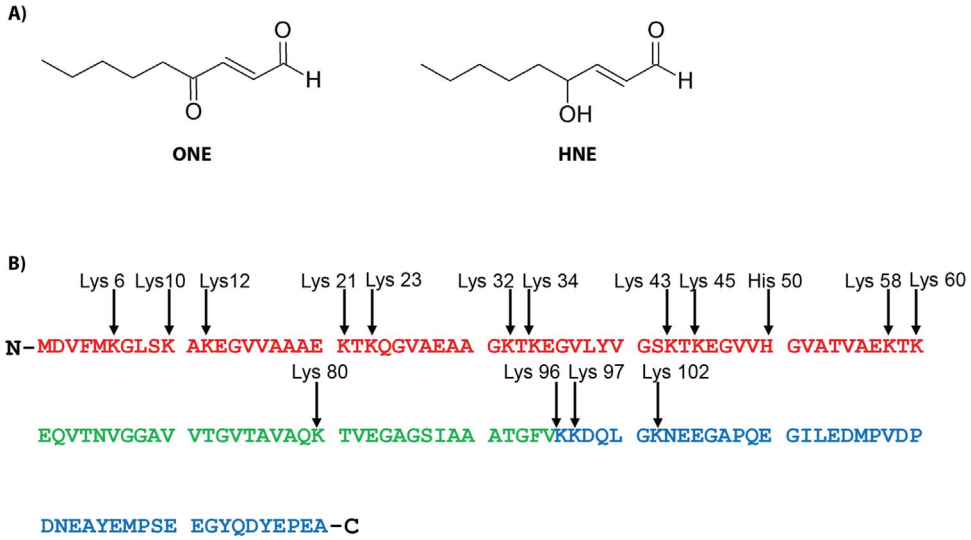

Fig. 1. The chemical structure of 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) (A). The alpha-synuclein amino acid sequence indicating His and Lys residues (B). Alpha-synuclein's three different structural regions: N-terminus (1–60, red), the hydrophobic core region (61–95, green) and the C-terminus (96–140, blue) (B).