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Research Article

Select polyphenols protect mitochondria against amyloid aggregates in Alzheimer and Parkinson diseases

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Abstract. Alzheimer and Parkinson diseases are age-related neurodegenerative disorders in which formation of amyloid aggregates by amyloid-beta (Abeta) and α-synuclein (αS) proteins, respectively, are recognised critical events that occur early in the disease process. These aggregates cause disruption of mitochondrial function in neurons, initiating a pathophysiological cascade leading to bio-energetic collapse and ultimately neuronal cell death. The detailed mechanisms are, however, largely unknown. In vitro studies in our laboratory aimed to, (i) investigate destabilisation of mitochondrial phospholipid membranes by these amyloid aggregates and, (ii) explore the protective effect of select polyphenolic compounds on mitochondria. Exposure of mitochondria, isolated from human neuroblastoma SH-SY5Y cells, to amyloid aggregates induced a strong and dose-dependent release of cytochrome *c*, reflecting damage to the outer and/or inner mitochondrial membranes. Importantly, targeting of aggregates to mitochondria was shown to be dependent upon cardiolipin, a mitochondria-specific phospholipid known to play a critical role in launching apoptosis. Moreover, the ability of amyloid aggregates to damage mitochondrial membranes was confirmed using a liposome permeabilisation assay. Finally, we found that the polyphenol compounds morin, rosmarinic acid, epigallocatechingallate and black tea extract were potent mito-protectants, and may thus delay the onset of neurodegenerative diseases.

Keywords Alzheimer's disease – Parkinson's disease – amyloid-beta – α-synuclein – mitochondrial membrane – permeabilisation – cardiolipin – polyphenols.

1 Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are incurable neurodegenerative disorders that are generally prevalent in the elderly population. Epidemiological studies in humans, as well as molecular studies in toxin-induced and genetic animal models of AD and PD show that mitochondrial dysfunction is a defect occurring early in the pathogenesis of both diseases (Büeler, 2009). Mounting evidence indicates that mitochondrial abnormalities triggered by small, soluble prefibrillar aggregates (termed oligomers) are early occurrences, preceding neurological pathology and clinical symptoms characteristic of both AD and PD. Perforation of mitochondrial membranes leads to the release of pro-apoptotic proteins such as cytochrome c (Cyto *c*) into the cellular cytosol, triggering neuronal apoptosis (Lin et al., 2009). In addition, epidemiological studies have associated nutrition components, such as polyphenols, to lower incidence of such neurodegenerative diseases, though the molecular basis for their protective effect is not known (de Lau and Breteler, 2006).

The aim of this paper is to describe *in vitro* studies that have been carried out in our laboratory which determine: (i) the effects of specific amyloid-beta (Abeta) and α-synuclein (αS) oligomeric aggregates on synthetic and cellular mitochondrial membranes, and (ii) identify mitochondrial-protective polyphenolic compounds. The first part of the report provides a background on the pathophysiological effects of such oligomeric proteins on mitochondria, followed by a brief description of the accomplished experiments.

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2 An overview of mitochondrial dysfunction in AD and PD

Mitochondria are organelles enclosed by a double membrane and are essential for cell viability. They are partitioned into four main compartments: the outer mitochondrial membrane (OMM), inter-membrane space (IMS), inner mitochondrial membrane (IMM) and matrix (Bogaerts et al., 2008). These organelles possess important functions within the cell, such as generation of adenosine triphosphate (ATP) during oxidative phosphorylation (Hausmann et al., 2008), homeostatic control of cytosolic calcium and iron concentration (Feissner et al., 2009), iron-sulfur (Fe/S) cluster and heme biogenesis (Lill and Mühlenhoff, 2005; Rouault and Tong, 2005) and also contribute to programmed cell death (Garrido and Kroemer, 2004). The critical role of a mitochondrial-specific phospholipid, cardiolipin (CL) in the apoptotic process has been extensively reported (Bradley et al., 2011; Korytowski et al., 2011). Cardiolipin is essentially an IMM phospholipid, since it is only present in minute quantities in the OMM, possibly through inner-to-outer mitochondrial membrane contact sites (de Kroon et al., 1997; Gonzalvez and Gottlieb, 2007). This phospholipid is critical for the optimal function of numerous enzymes that are involved in mitochondrial energy metabolism. Moreover, the formation of OMM openings required for the release of pro-apoptotic proteins such as Cyto *c* into the cytosol, was dependent on the presence of CL (Kuwana et al., 2002). While it is clear that some type of opening develops in the OMM during death signalling to allow exit of apoptogenic proteins from the IMS, the molecular nature and regulation of this pore remain unclear (Smith et al., 2005; Cheng et al., 2010).

2.1 Parkinson's disease

PD is distinguished from other forms of parkinsonism by the presence of Lewy bodies (LBs) and Lewy neurites (LNs), which are juxtanuclear and neuritic ubiquitinated protein aggregates composed predominantly of the synaptic protein αS (Shults, 2006). Cumulative evidence now suggests that the abnormal aggregation of this neuronal protein is critically involved in the pathogenesis of PD. The presence of mitochondrial dysfunction is noteworthy given the evidence already implicating mitochondrial toxins in the pathogenesis of PD (Camilleri and Vassallo, 2014). Sporadic forms of PD are attributed to environmental contaminants; substances like rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) inhibit complex I and also promote Cyto *c* release from the IMS (Perier et al., 2005; Banerjee et al., 2010). αS has been shown to interfere at various intracellular sites, including vesicle membranes (Iwai et al., 1995), mitochondria (Hsu et al., 2000), the cell death pathway (da Costa et al., 2006), the proteasome (Tanaka et al., 2001), and the Golgi apparatus (Gosavi et al., 2002). Although subcellular αS is largely cytosolic, a fraction of it was shown to be present in, or associated with, mitochondria (Nakamura et al., 2008). Devi and colleagues demonstrated import of αS into mitochondria and there it is shown to be predominantly associated with the IMM. This membrane association is due to the presence of a mitochondrial targeting signal and is dependent upon mitochondrial membrane potential, ATP levels and the oxidation of mitochondrial proteins (Devi et al., 2008; Parihar et al., 2008). Of interest is the fact that subcellular distribution of αS was found to be uneven between different brain regions, with higher levels of mitochondrial αS in the hippocampus, striatum and substantia nigra (Zhang et al., 2008).

A number of experimental studies suggest that αS may be closely linked to mitochondrial dysfunction and oxidative stress. An increase in the amount of αS binding with mitochondria was shown to be due to over-expression of αS in cell cultures (Shavali et al., 2008; Cole et al., 2008). Studies in yeast cells have demonstrated that abrogation of mitochondrial DNA prevented αS-induced reactive oxygen species (ROS) formation and apoptosis, implying that mitochondria are necessary in mediating the toxicity of αS (Büttner et al., 2008). Moreover, over-expression of A53T mutant αS was shown to induce Cyto *c* release and activation of downstream caspases in rat PC12 cells, resulting in apoptosis (Smith et al., 2005). Furthermore, association of αS to mitochondria in cells was also related to oxidation of mitochondrial proteins and elevated levels of calcium and nitric oxide (Parihar et al., 2008). The amount of αS associated with mitochondria seems to correlate with mitochondrial dysfunction.

Mitochondrial defects were also observed in numerous transgenic mouse models over-expressing wild-type (WT) or mutant αS. Some examples comprise selective oxidation of metabolic proteins related to mitochondria (Poon et al., 2005), evidence of mitochondrial pathology after treatment with MPTP (Song et al., 2004), deterioration of mitochondria containing αS including decreased complex IV activity and mitochondrial DNA damage (Martin et al., 2006). Worthy of note is the fact that some of the reported mitochondrial alterations due to expression of WT or A53T αS in mice were observed early in the disease process (Abou-Sleiman et al., 2006). These animal models suggest that αS may have a physiological role in mitochondria, and that the mitochondrial defects may be the result of a toxic gain-of-function due to over-expressed or mutated αS present in mitochondria (Büeler, 2009). In contrast, mice lacking αS expression also show mitochondrial dysfunction that is

manifested by altered plasma membrane lipid composition and reduced performance of the electron transport mitochondrial complex I and III activity (Ellis et al., 2005). As both the lack and overexpression of αS have adverse effects on neuronal mitochondria, the physiological equilibrium seems to be of pivotal importance.

2.2 Alzheimer's disease

AD is characterised by neuronal degeneration in the presence of extracellular plaque deposits of Abeta peptides in specific brain regions and intraneuronal, neurofibrillary tangles (NFTs) related to the hyperphosphorylation of the Tau protein in affected neurons (Murphy and LeVine, 2010). Mitochondria may be a common early target of both Abeta and Tau in AD. Gotz and coworkers reported an independent and synergistic action of both peptides on mitochondria leading to bioenergetic defects and increased oxidative stress in a triple transgenic mouse model exhibiting both pathological features of the disease (Eckert et al., 2010). The progressive mitochondrial accumulation of Abeta has been observed in the AD brain. Image analysis revealed accumulation of Abeta in 40 % and 70 % of mitochondria in the temporal lobe and hippocampus respectively, compared to 5 % and 14 % in non-AD brains (Chen and Yan, 2010). Abnormal mitochondrial gene expression, mitochondrial accumulation of Abeta and Abeta–dependent mitochondrial dysfunction were detected in AD transgenic mice from as early as 2-4 months of age, before the occurrence of cognitive difficulties (Reddy et al., 2004; Hauptmann et al., 2009; Du et al., 2010). In addition, mitochondrial dysfunction was observed to occur regardless of the genetic mutation involved, and lead to specific metabolic changes which could be used as biomarkers for the early diagnosis of AD (Trushina et al., 2012). These findings highlight the early and important involvement of mitochondria in the disease. Abeta-induced mito-toxicity is associated with decreased ATP production (Blass et al., 2002), increased ROS levels and oxidative stress (Rodrigues et al., 2001; Felice et al., 2007), disturbances of Ca^{2+} homeostasis (Sanz-Blasco et al., 2008), and destabilisation of mitochondrial membranes, amongst others. Nevertheless, the exact pathway and sequence through which these events occur remains unclear (Canevari et al., 2004).

To summarise, evidence suggests that mitochondrial dysfunction and intrinsic mitochondrial-mediated apoptosis play a key role in molecular cell death pathways of AD and PD (Northington et al., 2005; Martin, 2010). Several mechanisms of Abeta and αS-induced neurotoxicity have been proposed. Loss of normal cellular homeostasis and damage to cellular organelles such as mitochondria may indicate a unifying toxic cause: membrane permeation. Research reports support the hypothesis that Abeta and αS oligomers share a common mechanism of toxicity. Indeed, a common conformation unique to soluble oligomers has been identified (Glabe and Kayed, 2006), which specifically lead to permeabilisation of lipid bilayers regardless of protein sequence (Kayed et al., 2004). The importance of oligomer-induced mitochondrial impairments lies with their early and direct involvement in the pathogenesis of PD and AD (Cha et al., 2012; Trushina et al., 2012). Thus, drugs or natural compounds with the potential to adjust mitochondrial dynamics, function and biogenesis may help in attenuating the onset of both AD and PD.

3 Polyphenols as mito-protectants in vitro

Substantial evidence from the reviewed literature suggests that mitochondria are one of the main intracellular compartments linking the processes between the aggregation of Abeta and αS and final dopaminergic neuronal degeneration. Several natural polyphenolic compounds have been demonstrated to specifically and efficiently hinder the aggregation process of αS (Caruana et al., 2011). In our lab, we have also demonstrated the inhibitory effect of selected polyphenols and extracts on membrane damage, induced by αS and Abeta oligomers (Gauci et al., 2011; Caruana et al., 2012). On the basis of these results, we have extended our studies to investigate the destabilisation of synthetic and isolated mitochondrial phospholipid membranes by amyloid aggregates. The ultimate goal here was to establish whether mitochondrial membranes could be protected against amyloid-induced dysfunction by these promising groups of bioactive small-molecule compounds and extracts.

3.1 Amyloid aggregate-induced mito-toxicity

The first part of the study investigated the direct toxicity of Abeta and WT/mutant αS (A53T, A30P) aggregates on synthetic mitochondrial membranes. Liposome systems mimicking mitochondrial membranes have been used successfully in ascertaining the membrane permeabilising abilities of amyloid proteins (Epand et al., 2002; Kuwana et al., 2002; van Meer et al., 2008; Williams et al., 2010). The four types of synthetic lipid vesicle membranes used here mimic mitochondrial outer and inner membrane composition, as well as that of the neuronal plasma membrane for comparison (Table 1).

Assessment of vesicle permeabilisation by aggregated Abeta and WT/mutant αS was performed using fluorescence-based methods on such mitochondrial-like model systems. Essentially, the amyloid proteins were able to directly perforate the artificial mitochondrial membranes. We further noted that this effect was highly

Table 1: Phospholipid composition of IM-, OM- and L-type liposome membranes. OM-type liposomes - characteristic of mitochondrial membranes; IM-type liposomes - characteristicof inner mitochondrial membranes; L-type liposomes - characteristic of mitochondrial membranes but lacking cardiolipin.

Phospholipid	% composition		
	IM-type	OM-type	L-type
L-α-phosphatidylcholine (PC)	38	46.5	50.1
L-α-phosphatidylethanolamine (PE)	24	28.4	30.6
L-α-phosphatidylinositol (PI)	16	8.9	9.6
L-α-phosphatidylserine (PS)	4	8.9	9.6
Cardiolipin (CL)	18	7.3	-

dependent upon the presence of specific low-molecular-weight oligomeric species. In a further series of liposomal studies we discovered that mitochondria-specific lipid CL particularly enhanced permeabilisation by αS and Abeta (Chicco and Sparagna, 2007; Cole et al., 2008; Camilleri et al., 2013). Therefore it can be inferred from our experiments that IM-type membranes (rich in CL) are especially susceptible to Abeta and αS toxicity. Indeed, IM-membranes contain twice as much CL as OM-liposomes, whilst L-type liposomes (which lack CL) were the least damaged by the protein aggregates. Accordingly, it was suggested that mitochondria-localised αS is predominantly associated with the inner membrane in dopaminergic neuronal cell cultures and PD brains (Devi and Anandatheerthavarada, 2010). This supports recent data stating that oligomeric αS target disruption of lipid vesicles having negatively charged membranes and that CL is essential for the interaction between WT and A30P αS with large unilamellar vesicles whose composition is similar to that of the IMM (van Rooijen et al., 2009; Zigoneanu et al., 2012). In addition, it was reported that CL was the phospholipid found to most strongly stimulate Abeta aggregation (CL > PI > PS > PC = PE) (Chauhan et al., 2000) and that affinity of αS to CL possibly drives mitochondrial fission by αS oligomers, but not monomers (Nakamura et al., 2011). It is thus highly plausible that exclusive properties of the mitochondria-specific CL (see review by Szeto, 2014) provide a platform for rapid and enhanced membrane destabilisation by amyloid aggregates. These results indicate a pivotal pathway of neuronal apoptosis induction and intriguingly suggest a common toxic mechanism for the three aggregation-prone peptides.

An important endeavour of the study was to extrapolate the findings from the liposome permeabilisation assays to the membranes of respiring mitochondria isolated from a neuronal cell line. Interaction of aggregated Abeta and αS with the mitochondrial membrane lead to its disruption and release of the respiratory protein Cyto *c*, hence initiating mitochondrial-driven cell death pathways in neurons. For this purpose, a protocol was first established for isolation of fresh intact mitochondria from the SH-SY5Y human neuronal cell line. Subsequently, the isolated mitochondria were exposed to the proteins in question in order to determine whether this would damage the outer and/or inner mitochondrial membranes, specifically by inducing Cyto *c* release (CCR) from IMS. This was determined by means of a quantitative enzyme-linked immunosorbent assay. We reported that aggregated, but not monomeric, Abeta and WT/mutant αS disrupted the mitochondrial membrane leading to CCR. Taken together, the various *in vitro* neuronal models may indicate that amyloid misfolding is naturally paralleled to mitochondrial apoptosis and may contribute to neurodegeneration.

3.2 Polyphenols inhibit cytochrome *c* release from mitochondria

Polyphenolic compounds are naturally-present constituents of a wide variety of fruits, vegetables, food products and beverages derived from plants such as olive oil, tea, and red wine (Stevenson and Hurst, 2007). They are characterised by a polyphenol structure (Figure 1), which generally consists of two aromatic rings (2-phenyl-1,4-benzopyrone), each containing at least one hydroxyl group, which are connected via a three-carbon bridge and become part of a six-member heterocyclic ring (Beecher, 2003; Porat et al., 2006; D'Archivio et al., 2007). In the current literature, very few studies have to date examined the protective effect of polyphenols on amyloid-induced mitochondrial dysfunction. For instance, the flavone baicalein reduced the MMP-lowering effect of (E34K) mutant αS in whole cells (Jiang et al., 2010) and grape fruit extract protected mitochondria in a Drosophila model of PD (Long et al., 2009). Thus, it was relevant to investigate whether small-molecule polyphenols are able to suppress CCR from the IMS and therefore act as mito-protectants.

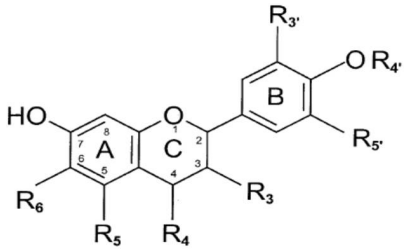

Figure 1: General structure and numbering pattern for common food polyphenols (adapted from (Beecher, 2003)). This figure shows the general structure and numbering pattern for common polyphenolic compounds. For most food flavonoids, R4′=H, R5=OH and R6=H. Individual flavonoids within each subclass are characterised by unique functional groups at R3, R3′, and R5′.

The relative efficacy of small-molecule compounds on preventing CCR from mitochondria was also determined by means of a quantitative enzyme-linked immunosorbent assay. The results demonstrated that the mitochondrial membrane can indeed be protected against αS/Abeta-induced disruption by a group of polyphenolic compounds, specifically by suppressing CCR. Seven polyphenolic compounds (apigenin, baicalein, epigallocatechin-gallate [ECGC], morin, nordihydroguaiaretic acid [NDGA] and rosmarinic acid [RA]) and black tea extract [BTE] were able to significantly limit CCR induced by aggregated WT/mutant αS and Abeta (Camilleri et al., 2013). It was encouraging to note that these compounds also featured as the top six compounds in the liposome permeabilisation assay. Hence, such experiments show that polyphenols constitute a major class of compounds that can indeed protect mitochondrial damage from αS and Abeta oligomeric assemblies at the membrane. Data emerging from our laboratory provides support to studies exploring the therapeutic potential of inhibitors of apoptosis proteins (IAPs) by raising the threshold of CCR required for commitment to apoptosis (Clayton et al., 2005).

4 Conclusion

It is increasingly evident that misfolded and aggregated disease proteins are not simply neuropathologic markers of neurodegenerative disorders but, instead, almost certainly contribute to disease pathogenesis. Our research was driven by the fact that formation of amyloid aggregates and mitochondria-induced apoptosis of neurons are critical events in the pathophysiology of these neurodegenerative diseases. Most importantly, mitochondrial dysfunction precedes neurological pathology and clinical symptoms characteristic of AD and PD.

Liposomal studies and CCR assays on isolated neuronal mitochondria in our laboratory clearly showed that specific proteins involved in AD and PD are able to permeate mitochondrial membranes and this ability is markedly dependent on aggregation conditions. Release of Cyto *c* suggests major disruption of mitochondrial membranes and is probably enough to induce neuronal apoptosis. An important finding underscored by liposomal studies is the prominent contribution of CL to both αS and Abeta-induced mitochondrial permeabilisation, accounting for the selective disruption of mitochondrial-like membranes as opposed to cellular-like membranes, and thus suggesting a common underlying mechanism of toxicity. Screening of a select group of small drug molecules and extract yielded potential mitoprotective agents with shared structural characteristics against toxicity induced by Abeta and αS aggregates. The *in vitro* assays performed in our studies are thus valid tools for the identification of potential therapeutic agents for subsequent *in vitro* analysis, hopefully arresting AD/PD at an early stage. In such debilitating maladies where no cure is available, increasing prevalence and high economic burden urgently necessitate disease-modifying treatment.

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