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Mitochondria and Alzheimer's disease: amyloid-β peptide uptake and degradation by the presequence protease, hPreP

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Abstract Several lines of evidence suggest mitochondrial dysfunction as a possible underlying mechanism of Alzheimer's disease (AD). Accumulation of the amyloid-β peptide (Aβ), a neurotoxic peptide implicated in the pathogenesis of AD, has been detected in brain mitochondria of AD patients and AD transgenic mouse models. *In vitro* evidence suggests that the Aβ causes mitochondrial dysfunction e.g. oxidative stress, mitochondrial fragmentation and decreased activity of cytochrome c oxidase and TCA cycle enzymes. Here we review the link between mitochondrial dysfunctions and AD. In particular we focus on the mechanism for Aβ uptake by mitochondria and on the recently identified Aβ degrading protease in human brain mitochondria.

Keywords Amyloid-β peptide · Alzheimer's disease · Presequence protease PreP · Aβ degradation · Mitochondria

Introduction

Alzheimer's disease (AD) is a complex disease and the most common age-related neurodegenerative disorder associated with neuronal death, dementia and ultimately death (Selkoe 2001; Mattson 2004). AD is characterized by extracellular amyloid plaques, mainly consisting of the hydrophobic 40–42 amino acid long amyloid-β peptide (Aβ) (Glenner and Wong 1984) and intracellular neurofibrillary tangels composed of aggregated hyperphosphorylated tau protein (Nukina and Ihara 1986). There are several different theories explaining why neuronal cell death occurs in this disease. However, the major hypothesis is the amyloid cascade hypothesis (Hardy and Higgins 1992). According to this theory, a distorted Aβ metabolism, production and/or degradation, triggers aggregation of Aβ peptide causing synaptic deficits, neurofibrillar tangles, inflammatory response, elevated oxidative stress, cell death and eventually AD. Aβ is a proteolytic product of the sequential cleavage of the amyloid precursor protein (APP) by groups of enzyme complexes termed β- and γ-secretases (Vassar et al 1999; Kimberly and Wolfe 2003). It was first believed that amyloid plaques were toxic and caused neurodegeneration. However, opinion has changed towards considering Aβ oligomers as the most toxic species (Hardy and Selkoe 2002). Notably, a growing number of reports suggest that beside its well characterized neuropathological symptoms, AD is also believed to be associated with many intracellular lesions such as perturbation of Ca^{2+} homeostasis, accumulation of Aβ in the secretory pathways and the presence of Aβ in mitochondria leading to mitochondrial dysfunction and elevated reactive oxygen species (Manfredi and Beal 2000; Gouras et al. 2005; Reddy and Beal 2005; Anandatheerthavarada and Devi 2007; Reddy and Beal 2008). Mitochondria, the ATP generating organelles and the key regulator of cell death have therefore also recently been implicated in AD. Two mitochondrial proteins, Aβ-binding alcohol dehydrogenase (ABAD) and cyclophilin D (CypD) have been suggested to be associated to this neurodegenerative disorder (Lustbader et al. 2004; Du et al. 2008). Here we will review and discuss the link between mitochondria and AD, especially the mechanism behind Aβ uptake by the organelle and the

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degradation of this toxic peptide by the recently identified mitochondrial Aβ-degrading protease, PreP.

Mitochondrial dysfunctions and AD

The brain requires a high amount of energy for neurotransmission and therefore mitochondria are highly enriched at synapses for generation of ATP. Notably, a reduction in the number of mitochondria and a decreased energy metabolism are among the earliest detectable defects in AD brains (Hirai et al. 2001; Mosconi et al. 2005). A decreased activity of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) in the frontal and temporal lobe in AD brains has been demonstrated and the reduced enzymatic activity was not correlated with altered protein expression (Sorbi et al. 1983; Gibson et al. 1998). Also, the activity of cytochrome c oxidase (complex IV) of the electron transfer chain has been shown to be diminished in AD brain mitochondria (Kish et al. 1999; Cardoso et al. 2004). Since the function of all of these enzymes is inhibited in the presence Aβ, a link between the amyloid cascade theory and mitochondrial dysfunction in AD has been proposed (Casley et al. 2002).

Moreover, APP has been shown to harbour a chimeric targeting signal consisting of an N-terminal hydrophobic endoplasmic reticulum (ER) followed by a mitochondrial targeting signal (Anandatheerthavarada et al. 2003; Devi et

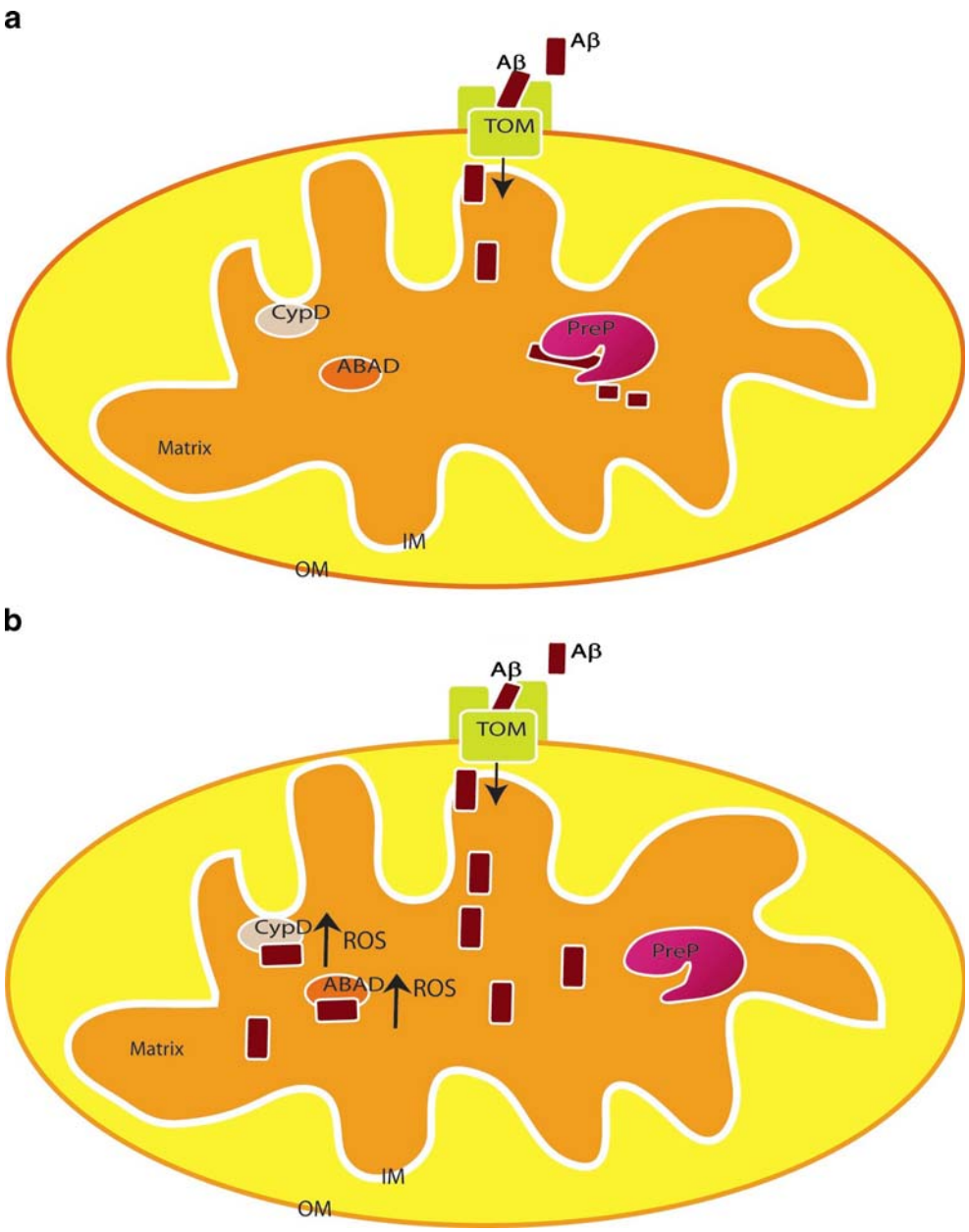

Fig. 1 Hypothetical role of hPreP in detoxification of mitochondria in Alzheimer's disease. Aβ is taken up by mitochondria via the TOM complex and the imported Aβ resides preferentially in the inner membrane. However, Aβ can also reach the matrix, where it can be degraded by hPreP (**A**). Under conditions when Aβ is not degraded by hPreP, it interacts with CypD and/or ABAD causing elevated ROS production and cell toxicity (**B**)

al. 2006). A recent study demonstrated that APP is arrested and accumulated in the Translocase of the outer membrane (TOM) machinery of human AD brain mitochondria probably due to the presence of an acidic region between residues 220–290 that prevents full translocation into mitochondria. This suggests that the N-terminal portion of APP is inside the organelle leaving the Aβ containing portion in the cytosol. The accumulation of APP blocked the mitochondrial import pore in AD brain preventing import of mitochondrial precursor proteins. Furthermore, an early mitochondrial dysfunction associated with elevated reactive oxygen species (ROS) production, decreased mitochondrial membrane potential, ATP level and complex IV activity was detected in transgenic mice overproducing APP (Hauptmann et al. 2008). A recent report has shown that overexpression of APP leads to fragmentation of mitochondria and abnormal mitochondrial distribution in neuroblastoma cells as well as in rat primary neurons. However, in the presence of a β-secretase inhibitor the production of Aβ was reduced and the fragmentation was rescued demonstrating that the fragmentation is due to overproduction of Aβ (Wang et al. 2008).

Importantly, the accumulation of Aβ in affected AD brain mitochondria as well as in the brain mitochondria of transgenic mice overproducing mutated APP (Tg mAPP mice) has been shown (Lustbader et al. 2004; Caspersen et al. 2005; Manczak et al. 2006). In the Tg mAPP mice, the mitochondrial Aβ accumulation arises at around 4 months, before formation of plaque (Caspersen et al. 2005). Furthermore, ABAD, a mitochondrial matrix localized short chain alcohol dehydrogenase with an essential physiological role in mitochondria, has been found to be up-regulated in the AD temporal lobe as well as in Tg mAPP mice in comparison to age-matched control cases (Yan et al. 1997; He et al. 2002; Wen et al. 2002). It has been shown that ABAD specifically binds Aβ inside the mitochondrial matrix of AD brains and transgenic mice overexpressing both APP and ABAD (Tg mAPP/ABAD) (Lustbader et al. 2004). The Aβ-ABAD interaction caused elevated ROS production and cell death in neuronal cultures from these mice. In addition, increased oxidative stress and memory deficits were detected in 5 month old Tg mAPP/ABAD mice. The crystal structure of ABAD-Aβ has been determined demonstrating that Aβ binds to the NAD^+ binding pocket and prevents the binding of the cofactor to ABAD thereby inhibiting the activity of ABAD, which causes mitochondrial dysfunction. In addition, it has been shown that Aβ specifically interacts with CypD, a mitochondrial matrix protein, which upon its association with mitochondrial permeability transition pore (mPTP) in the inner membrane promotes opening of mPTP causing cell death. The interaction of CypD with mitochondrial Aβ potentiates free radical production in neurons and synapsis and also promotes opening of mPTP leading to apoptosis (Du et al. 2008). In CypD knockout mice, the neurons are protected from Aβ and oxidative stress induced cell death.

Aβ uptake by mitochondria

Data generated from brain biopsies taken from living patients with normal pressure hydrocephalus harbouring plaque pathology also had Aβ42 localized in their mitochondria. Aβ42 was not found in brain mitochondria of patients who lacked plaque pathology. In addition, extracellularly applied Aβ can be taken up by neuroblastoma cells and ends up in mitochondria (Hansson Petersen et al. 2008). The incomplete import of APP into mitochondria, leaving the Aβ-region outside the membrane (Anandatheerthavarada et al. 2003), indicates that Aβ cannot be generated inside mitochondria and therefore has to be imported into the organelle. There are different channels and translocases located in the mitochondrial outer membrane that can mediate the passage of Aβ, such as the TOM machinery responsible for import of mitochondrial precursor proteins, the voltage-dependent anion channel (VDAC), which permits molecules up to 8 kDa to cross the membrane, and the above mentioned mPTP. We have investigated the pathway behind the uptake of Aβ40 and Aβ42 by mitochondria using an *in vitro* import assay, in which isolated rat liver mitochondria were incubated with Aβ40 and Aβ42 in the absence or presence of antibodies directed to the TOM components or VDAC and the inhibitor of mPTP, cyclosporine A. The import of Aβ peptides was not abolished in the presence of antibodies against VDAC or in the presence of cyclosporine A. However, in the presence of antibodies raised against subunits of the TOM complex, i.e. Tom20, Tom70 (the protein import receptors) or Tom40 (the import pore), the import of Aβ peptides was prevented showing that the Aβ peptides are transported into mitochondria through the TOM machinery. Since Aβ is very hydrophobic in the C-terminal portion it can create pores in a membrane itself (Bezprozvanny and Mattson 2008). Elimination of Aβ uptake after pre-shaving of mitochondria before the import reaction ruled out the possibility of an unspecific Aβ association with the mitochondrial membrane and also further proved the important role of the TOM machinery for the uptake of Aβ. Import was not affected by the addition of valinomycin, an ionophore, which disrupts the membrane potential, indicating that import is membrane potential independent. After being taken up by the organelle, Aβ was mostly found to be localized in the mitochondrial cristae and associated with the inner membrane fraction. Also, immunoelectron microscopy analysis data of brain biopsies from living patients with plaque pathology displayed most of the

Aβ to be associated with the inner membrane. In post-mortem AD brains, Aβ was found in the matrix fraction (Caspersen et al. 2005). In the *in vitro* assay, mitochondria are incubated with Aβ for 30 min and it is possible that Aβ does not reach the matrix or that it is degraded by the recently found Aβ-degrading protease, PreP, which is thoroughly discussed below. During the development of AD, Aβ might be associated to ABAD or CypD (Lustbader et al. 2004; Du et al. 2008) and can no longer be degraded by PreP and can therefore be detected in the matrix of post-mortem AD brains.

Degradation of Aβ by hPreP

We have identified an Aβ-degrading enzyme in mitochondria, called the Presequence Protease, PreP. PreP is a metalloprotease containing an inverted zinc-binding motif (HXXEH) and belongs to the pitrilysin oligopeptidase subfamily (subfamily M16). This protease was initially found and characterized in *Arabidopsis thaliana* as the enzyme responsible for degradation of targeting peptides, presequences, which have been cleaved off inside mitochondrial matrix after protein import, but it is also in charge of cleaning the organelle from other unstructured peptides up to 65 amino acids (Stahl et al. 2002, 2005; Moberg et al. 2003). Interestingly, PreP is an organellar functional analogue of human insulin degrading enzyme (IDE), which also belongs to pitrilysin family. IDE is implicated in AD due to its ability to degrade Aβ (Mcdermott and Gibson 1997; Morelli et al. 2004). hPreP was originally identified as human metalloprotease 1, hMp1 (Mzhavia et al. 1999), which consists of 1,037 amino acids (AAH05025) and is encoded by the *PITRM1* gene located on chromosome 10. Bioinformatic prediction programs predicted hPreP to be a mitochondrial protein and our intra-mitochondrial localization studies demonstrated PreP to be localized in the mitochondrial matrix in mammals. In addition, proteome studies of human mitochondria have confirmed the mitochondrial matrix localization of hPreP (Taylor et al. 2003). Importantly, we were able to show that hPreP is the sole protease responsible for degradation of Aβ in mitochondria since immuno-inactivation studies *in situ* using anti-hPreP antibodies abolished the activity against Aβ (Falkevall et al. 2006). hPreP is able to completely degrade Aβ40 and Aβ42 as well as the Arctic Aβ40 (E22G), the peptide that causes AD-like pathology, in an ATP independent manner. The degradation pattern of Aβ40 and Arctic Aβ40, analysed by LC-MS/MS, resulted in the production of several fragments after cleavage at sites, such as Gln15↓Lys16, Lys16↓Leu, Ala30↓Ile31, Gly33↓Leu34 and Leu34↓Met35 that are unique for hPreP. Interestingly, a number of the cleavage sites are located after the Gly29 in the very hydrophobic C-terminal portion of the peptides that is prone to aggregation. Unlike IDE, PreP cannot degrade insulin. IDE harbours an exosite in the catalytic chamber, which is hypothesized to unfold small proteins (Shen et al. 2006). The corresponding site is absent in the PreP structure making PreP incapable of degrading small folded proteins. This fact alone makes PreP a better candidate than IDE for clearing up Aβ since it cannot degrade an important regulating protein such as insulin.

Molecular homology model of hPreP based on the solved 3D structure of *At*PreP (Johnson et al. 2006) showed that it consist of 4 domains, creating two halves that are connected by a hinge region. The two halves can come together creating a large catalytic chamber of 10 000 $Å^3$. The inverted zinc-binding motif is located in the N-terminal portion, but the residues located in the C-terminal half, about 800 amino acids distant from the zinc-binding motif, complete the active site. Functional analysis of single nucleotide polymorphism variants of hPreP (hPreP-SNPs) showed a dramatically decreased activity for the hPreP(A525D) variant with a mutation situated in the hinge region (Bjork B. *et al* unpublished). This region is hypothesized to be of importance for the opening and closing of the proteolytic chamber. Hence, we believe that uncommon substitution in hPreP may contribute to less efficient clearance of Aβ and other toxic peptides in mitochondria, which thereby may contribute to mitochondrial dysfunctions.

To our surprise we discovered two cysteines in the homology model of hPreP, Cys90 in the N-terminal portion and Cys527 located in the hinge region, to be in close vicinity to each other. These cysteines are conserved in all mammalian PreP sequences. Interestingly, measuring the proteolytic activity of hPreP under oxidizing conditions demonstrated an abolished activity against Aβ, pointing towards a disulphide bridge formation between Cys90 and Cys527 that locks the enzyme in a closed conformation and inhibits the substrate from entering the catalytic chamber. The involvement of these cysteines in creating disulphide bridges and causing inhibition of hPreP function was confirmed by demonstrating full proteolytic activity of the hPreP(C90S) and hPreP(C527S) variants under oxidizing conditions. These findings indicate a possible inhibition of hPreP under elevated ROS production in mitochondria implicated in AD, and might therefore be of physiological importance in AD. In addition, it is possible that the binding of Aβ to ABAD or CypD (discussed above) might be a result of inactivation of hPreP in AD patients (Fig. 1).

In summary, accumulating evidence implicates mitochondrial dysfunctions in AD. Approaches to protect mitochondria from Aβ may prevent the neuropathology in patients suffering from this disorder. Accumulation of Aβ in the brain of AD patients and its binding to mitochondrial proteins such as CypD and ABAD causes oxidative stress,

damaged mitochondrial function and apoptosis. We have recently demonstrated mitochondrial uptake of Aβ via the TOM complex and showed that PreP is the protease responsible for Aβ degradation inside mitochondria. Avoiding accumulation of Aβ inside mitochondria and preventing the binding of this toxic peptide to mitochondrial proteins either by inhibition of Aβ uptake or enhancing Aβ clearance by PreP might thereby rescue mitochondrial dysfunctions and elevated ROS production and abolish neuronal death.

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