Mitochondrial β-amyloid in Alzheimer's disease

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Abstract: It is well established that the intracellular accumulation of beta-amyloid is associated with Alzheimer's disease and that this accumulation is toxic to neurons. The precise mechanism by which this toxicity occurs is not well understood, however, identifying the causes of this toxicity is an essential step in developing treatments for Alzheimer's disease. One intracellular location where the accumulation of beta-amyloid can have a major effect is within mitochondria has identified mitochondrial proteins that act as binding sites for beta-amyloid and when binding occurs a toxic response results. For one of these identified sites, an enzyme known as 'ABAD', we have identified the changes in gene expression in the brain cortex following beta-amyloid accumulation within mitochondria. Specifically, we have identified two proteins that are upregulated in the brains of transgenic animal models for Alzheimer's disease but also human sufferers. The increased expression of these proteins demonstrates the complex and counter-acting pathways that are activated in Alzheimer's disease. Previous studies have identified the approximate contact sites between ABAD and beta-amyloid, and based on these observations we have shown that using a modified peptide approach, it is possible to reverse the expression of these two proteins in living transgenic animals and also recover both mitochondrial and behavioural deficits. This indicates that the ABAD-beta-amyloid interaction is potentially an interesting target for therapeutic intervention. To explore this further we used a fluorescing substrate mimic to measure the activity of ABAD within living cells, and in addition we have identified chemical fragments that bind to ABAD, by using a thermal shift assay.

Keywords: Alzheimer's disease, mitochondrial dysfunction, intracellular Aβ, ABAD, CHANA, cyclophilin D,

Abbreviations used:
Aβ, amyloid-beta peptide; ABAD, amyloid-binding alcohol dehydrogenase; AD, Alzheimer's disease; ANT, adenine nucleotide translocase; Cdk5, cyclin-dependent kinase 5; CHANA, cyclohexenyl amine naphthalene alcohol; CHANK, cyclohexenyl amine naphthalene ketone CypD; cylcophilin D; DP, decoy peptide; EFHD 2, EF-hand domain containing protein 2; ELISA, enzyme linked-immunosorbent assay; Ep-1, endophilin-1; ER, endoplasmic reticulum; ERAB, ER associated amyloid binding protein; JNK, c-Jun-N-terminal kinase; mAPP, mutant amyloid precursor protein; the mitochondrial permeability transition pore mPTP; PiC, mitochondrial phosphate carrier; Prx-2, peroxiredoxin-2; ROS, reactive oxygen species; SPR, surface Plasmon resonance; TOM, translocase of outer mitochondrial membrane; VDAC, voltage-dependent anion channel

Introduction

A link between mitochondrial dysfunction and neurodegenerative conditions such as Alzheimer's disease (AD), has long been suggested. Observations in dementia patients of altered cerebral blood flow and bioenergetic deficits in dementia patients using positron electron tomography (*1-2*) initiated research into the function of mitochondria in neurodegeneration, as they are central to cellular energy metabolism. Evidence has since accumulated for the pivotal role of metabolic pathways and free-radical turnover in mitochondria during both normal aging and neurodegenerative diseases (*3-4*). For example, Parker and colleagues detected reduced cytochrome C oxidase activity in the platelets (*5*) and the brains of AD patients (*6*) while Sayre et al. found increased levels of 4-hydroxynonenal (4-HNE), a product of lipid peroxidation, in AD brain tissue compared to controls (*7*). Due to the proximity of mitochondrial DNA to reactive oxygen species (ROS), which are naturally produced in the electron transport chain, this DNA is thought to accumulate mutations over time, which can lead to increasing mitochondrial dysfunction and increased production of ROS with age (*8-10*). Key mitochondrial enzymes, that are affected by AD include cytochrome C oxidase (*5*, *11*), the pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase and the isocitrate-dehydrogenase (*12-13*).

Lustbader *et al.* used immunoelectron microscopy to show that mitochondrial β-amyloid (Aβ) (both the 1-40, and 1-42 forms) localises inside the mitochondria of both mutant anyloid precursor protein (mAPP) over-expressing transgenic animals (Tg mAPP) and, significantly, the human AD brain (*14*). A further study showed that Aβ accumulation in the mitochondria from Tg mAPP mice and the cerebral cortex of human AD brains is significantly higher than in non-transgenic mice and non-AD brains (*15*). More recent studies, indicate that mitochondrial dysfunction and the accumulation of mitochondrial Aβ (and ABAD: see below) can be observed in the early disease stages of AD in other commonly used transgenic animal strains, including the popular triple transgenic mouse model (human APP_{SWE}, Tau_{P301L}, and $PS1_{M146V}$;(*16*)).

The origin of this mitochondrial Aβ is still under debate and there is experimental evidence for both the local production (for example the presence of the γ-secretase complex within mitochondria) and/or the import of Aβ from the cytosol via the translocase of outer mitochondrial membrane (TOM) (*17-19*). In addition to the ongoing efforts to identify how Aβ occurs within mitochondria, the other key question regards the action of Aβ once it is located within mitochondria. The addition of Aβ to cell cultures induces dysfunction of mitochondrial respiration, ATP depletion and production of ROS (*20-21*) and exposure of isolated mitochondria to Aβ reduces complex IV activity (*22*) and induces the formation of the permeability transition pore, which is linked to cell death (*23*). In addition to these organelle-level changes, within the last decade attention has increasingly focused on two mitochondrial proteins, amyloid binding alcohol dehydrogenase (ABAD) and Cyclophilin D (CypD), both of which appear able to mediate the toxicity of the Aβ peptide. Binding of both proteins to Aβ (both the 1-40 and 1-42 forms) has been demonstrated at nM Aβ concentrations, and Aβ accumulation within cells is known to result in an increase in expression of both of these proteins (*23-25*).

Cyclophilin D

CypD, a peptidylprolyl isomerise F, is found in the mitochondrial matrix and translocates to the inner mitochondrial membrane during times of oxidative stress where it is thought to play a role in the opening of the mitochondrial permeability transition pore (mPTP) (*26*). CypD is considered to be a part of the mPTP as it associates with adenine nucleotide translocase (ANT) and possibly other factors, like the voltage dependent anion channel (VDAC) and the mitochondrial phosphate carrier (PiC) to contribute to the opening of the pore (*27*). Using a variety of methods such as immunoprecipitation, co-localisation and surface plasmon resonance (SPR) assays it was shown that CypD can bind to Aβ at nM concentrations of Aβ, However, no direct contact sites have yet been identified (*23*). Du et al. (*23*) also reported that under Aβ rich conditions in the aging brain, CypD expression levels are increased and coincide with increased levels of ROS production. Further studies using Tg mAPP mice deficient in the gene encoding CypD revealed that the interaction of CypD with Aβ can result in cellular stress and cell death (*23, 25*). Notably, neurons derived from CypD deficient animals are resistant to Aβ-induced opening of the mPTP, and are thus protected against Aβ- and oxidative stress-induced cell death. These animals also exhibited significantly improved learning and memory function when compared to transgenic mAPP mice with normal expression of CypD (*25*).

Amyloid binding alcohol dehydrogenase (ABAD)

ABAD is the most characterised intracellular Aβ-binding protein and was first identified in 1997 using a yeast two-hybrid screen (*24*). It was originally identified within the endoplasmic reticulum (ER) and termed ERAB (ER associated amyloid binding protein) (*24*), however, later studies confirmed its presence inside mitochondria (*28*). The action of this enzyme is primarily to catalyse the reduction of aldehydes and ketones or the reverse reaction of oxidation from alcohols for energy production. As described in more detail by Muirhead et al. (2010) (*29*) ABAD acts on a variety of substrates, indicating the variety of functions it can have within the cell. This variety of potential substrates correlates with the finding that ABAD appears to act as a molecular switch. In the presence of low levels of Aβ ABAD can have neuroprotective effects, and its increased expression is protective in animal models for Parkinsonism (*30*) and metabolic stress (*31*); however, as Aβ levels rise, it appears that ABAD loses its ability to protect and enhances Aβ toxicity (*32*). X-ray crystallography studies of both human and rat ABAD have provided a clear representation of the catalytic core of ABAD and enabled the identification of key residues involved in substrate binding and the interacftion with Aβ (*33*). However the precise residues involved in the Aβ-ABAD interaction could not be indentified by crystallography as loop D, the region that is thought to bind Aβ, was disordered in the structure (*14*). The interaction with Aβ not only inhibits ABAD's enzyme function (though notably only at μM concentrations) (*28, 34*), but importantly also causes severe mitochondrial dysfunction and cellular toxicity (*14, 32*), which cannot be attributed to a loss of enzyme function alone. Proteomics studies on mice over-expressing ABAD and mAPP revealed that in the living brain the ABAD-Aβ interaction also affects the expression of proteins. Those proteins specifically identified were Endophilin-1 (Ep-1) (*35*) and Peroxiredoxin-2 (Prdx-2) (*36*), both of which were found to be more abundant in human AD brains compared to controls (*35-36*). To date, the link between these two proteins and mitochondrial dysfunction remains unclear. However, evidence exists that increased levels of Ep-1 can cause the activation of c-Jun-N-terminal kinase (JNK) (*35, 37*). JNK is a stress kinase which has been linked to Aβ production in neuronal cells (*38-39*) and the action of which is associated with the early stages of AD (*40*). Conversely, Prdx-2 is an anti-oxidant protein and an increase in its expression can protect against Aβ-induced toxicity (*36*). Thus the increased expression of these two proteins typifies the competing pathways that are activated in the AD diseased brain.

Another important metabolic function located in mitochondria is Ca^{2+} homeostasis. Evidence for disturbed Ca^{2+} homeostasis (*41*) and alterations in Ca^{2+} regulated proteins, especially the neuronal proteinase calpain and its targets, have been detected in human AD brains, in human cortical neuron cultures *in vitro* (*42-43*) and transgenic mice (*44*). Interestingly, a link between the calpain-regulated cyclin-dependent kinase 5 (Cdk 5) and Prdx-2 inactivation by phosphorylation has been discovered in a model for Parkinson's disease (*45*). In addition, Cdk 5 had been earlier identified as a candidate kinase for mediating neuronal toxicity in AD (*46-48*). The relevance of this pathway for Prdx-2 function in AD has not been investigated so far. Our own studies indicate that the phosphorylation of Thr89 in Prdx-2 can regulated the observed protection against Aβ-induced toxicity (Borger, unpublished observations). More recently a novel Ca^{2+} binding protein, EFHD 2 (swiprosin 1) has also been linked to one of the hallmarks of AD (*49*). EFHD 2 is more associated with hyperphosphorylated tau protein in a mouse tauopathy model and in human AD brains, than in control tissues (*49*). Additionally, it was reported that the protein levels of EFHD 2 were increased in AD cases compared to controls (*49*) and our own

studies suggest that this is also the case in the Tg mAPP mouse model, which does not develop tauopathy (Borger, unpublished results). The function of EFHD 2 in neuronal cells is still unknown, but its association with two of AD hallmarks points towards a potential importance in AD pathology.

Taken together, research on human dementia patients and studies using animal as well as *in vitro* models for AD have shown that all the key functions of mitochondria are affected in AD. A detrimental link between impaired brain energy metabolism, ROS production and disturbed Ca^{2+} homeostasis has also been established for other neuropathological conditions such as delirium, ischemia and hypoglycaemia. Consequently, Blass and colleagues have proposed a role for a downward "mitochondrial spiral" in the development of neurodegenerative diseases, in particular for AD (*50*-*52*).

Therapeutic targets and development of assays

The proposed binding site of Aβ on ABAD, "loop D", was identified through a range of complimentary techniques including X-ray crystallography and mutagenesis studies (*14*). It has been shown that a synthetic peptide consisting of residues 92-120 which form this loop can be used as a "decoy peptide" (DP), which competes with ABAD as a binding partner for Aβ. Using SPR, it was shown that this "decoy peptide" was able to prevent the binding of Aβ to ABAD (*14*). The same region of ABAD, identified independently through an antisense peptide approach, was found to bind biotinylated Aβ with a K_d of 107 nM using ELISA (*53*). Cellular studies of the DP effects, using a Tat-DP fusion peptide to allow the peptide to cross the cell membrane, found that application of the peptide was protecting neurons (from wild type, Tg-ABAD and Tg-ABAD/mAPP mice) against Aβ-induced toxicity (*14*). Similarly, introduction of DP-TRX (TRX = thioredoxin 1, an enzyme introduced here in order to stabilise the peptide) into cell cultures using a lentiviral approach revealed that DP-TRX-transfected cells showed decreased apoptosis, decreased LDH release and increased cell viability after treatment with Aβ compared to controls (*54*). Compelling evidence for the effectiveness of the peptide came from *in vivo* studies in transgenic animals, where a fusion peptide consisting of amino acids 93-116 of loop D with a Tat sequence and a mitochondrial targetings sequence (Tat-mito-DP(93-116)) was found to alter levels of the AD biomarker proteins Prdx II and Ep I (*35*-*36*). Intraperitoneal injection of Tat-mito-DP(93-116) into Tg mAPP mice resulted in a reduction of Prdx II levels in these mice to levels comparable with non-transgenic mice (*36*). Similarly, Ep I levels were elevated in double transgenic ABAD/ mAPP mice and were found to return to basal levels when the mice were treated with Tat-mito-DP(93-116) (*35*). In addition, behavioural studies revealed that treatment with the DP improved short-term memory performance in transgenic AD mouse models. Recently Yao et al. demonstrated, that both double transgenic mAPP/Tat-mito-DP(91-119) mice as well as mAPP mice systemically treated with the decoy peptide showed significant improvement in the radial-arm water maze test for short-term memory performance compared with untreated mAPP mice (*55*).

In order to develop therapeutic compounds that target the Aβ-ABAD interaction, a robust method for monitoring the activity of the desired drug target is required. The use of purified ABAD protein to measure enzyme activity *in vitro* is well documented (*29*). Utilising the absorption of NADH at 340 nm, the rate of reduction of substrate can be measured by spectroscopy. Recently we and our collaborators have developed a cell-based assay that enables the study of ABAD activity in intact living cells. The assay uses a fluorescent ABAD substrate mimic,

cyclohexenyl amine naphthalene alcohol (CHANA), which was based on the endogenous ABAD substrate oestradiol. CHANA is a non-fluorescent substrate under the assay conditions, while the reaction product cyclohexenyl amine naphthalene ketone CHANK fluoresces in non-aqueous environments, e.g. in cell membranes (*56*). Due to these different photochemical properties, selective detection of the accumulation of CHANK is possible. Thus the conversion of CHANA to CHANK, and hence ABAD activity, can be monitored by fluorescence microscopy. This novel approach has recently been improved by synthesis of the individual stereoisomers of CHANA. (–)-CHANA was found to be selectively metabolised by ABAD, while (+)-CHANA showed a much higher level of background oxidation by other cellular dehydrogenases (*57*). Thus, using (–)-CHANA, we have laid the foundations for a cell-based assay monitoring ABAD activity. Using (–)-CHANA metabolism was seen to be substantially diminished in cells treated with a known ABAD inhibitor, AG18051 (*33*). Importantly, it was also demonstrated that Aβ can inhibit ABAD activity in living cells. Upon addition of 22 μM Aβ(1-42), HEK293 cells were shown to exhibit a significant decrease in (–)-CHANA metabolism of around 20%. It is hoped that further development of this system will provide a robust cell-based assay for use in identifying potential modulators of ABAD function (*57*).

The Future

AD is a complex disease, and likewise it becomes more and more apparent that the toxicity of the Aβ peptide is also a complex process. Indeed whilst many approaches to explain AD pathology have centred on extracellular Aβ, there has been less focus on the involvement of intracellular species of Aβ. Our studies with our collaborators have focussed on these events (summarised in Figure 1). They suggest that the one drug and one target approach for the treatment of AD are unlikely to be an effective strategy for the treatment of AD. Consequently, the treatment of this disease will likely require a three armed approach, targeting for example: 1) The formation/ clearance of Aβ and hyperphosphorylated tau; 2) The support and stabilisation of the remaining neuronal networks and 3) Protection of potential sensitive intracellular targets. The integrity and function of mitochondria is one such sensitive intracellular target, and though ABAD and CypD are not classical drug targets they are important players in the sequence of events resulting in mitochondrial dysfunction. Therefore, future treatment approaches to the treatment of AD will greatly benefit from understanding their involvement in AD.

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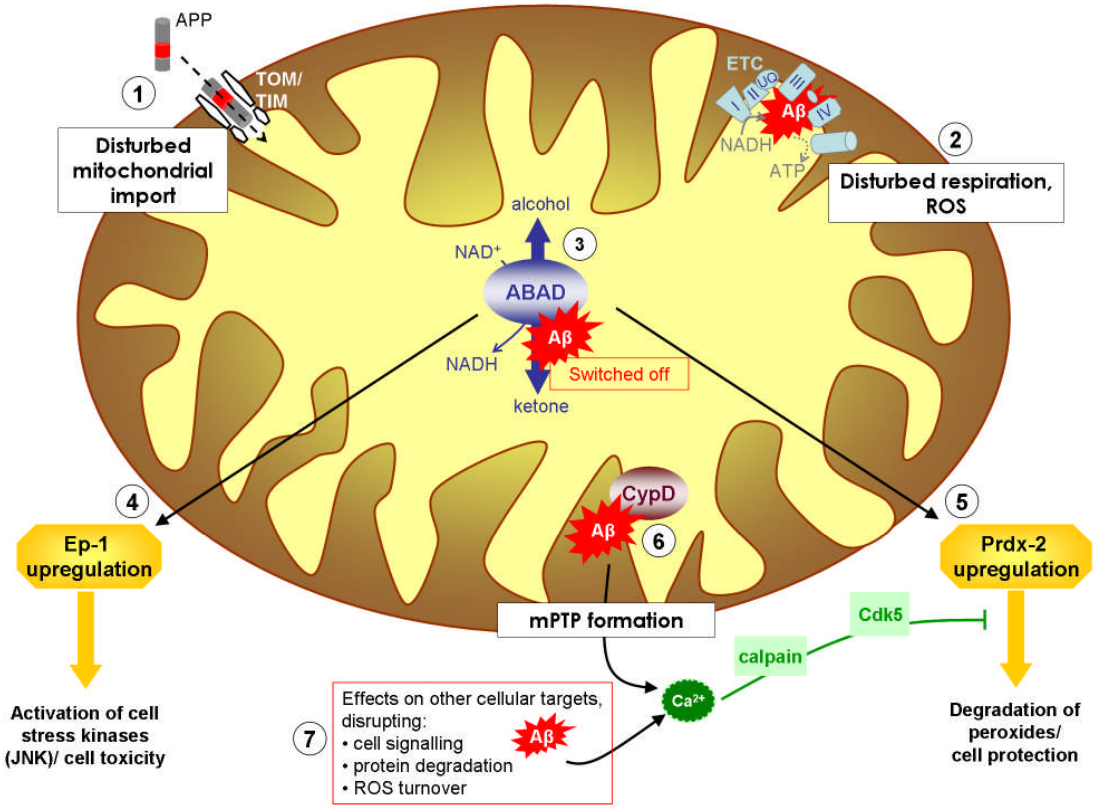

Figure 1: Consequences of mitochondrial Aβ

APP can be transported to mitochondria, where it interacts with TOM and TIM (transolcase of the inner membrane), disturbing mitochondrial protein import (1). Aβ can be imported into mitochondria via TIM and TOM and is found associated with the inner mitochondrial membrane, disrupting mitochondrial respiration and leading to excess production of ROS (2). Aβ has been found to interact with ABAD in the mitochondrial matrix, inhibiting the enzyme (3). The Aβ-ABAD interaction also leads to an up-regulation of AD biomarkers Ep-1 (4) and Prdx-2 (5). At the inner mitochondrial membrane, Aβ can with CypD, which is involved in the formation of the mPTP and Ca^{2+}-relase from mitochondria (6). Aβ can be found in the cytosol, disturbing cell signalling, protein degradation and causing ROS production, which leads to an increase in cytosolic Ca^{2+} (7). Ca^{2+} mediated Cdk5 activation has been found to inhibit the Prdx II antioxidant function.