Amyloid-β Peptide Induces Mitochondrial Dysfunction by Inhibition of Preprotein Maturation

Dirk Mossmann, 1,2,3,14 F.-Nora Vogtle, 1,14 Asli Aras Taskin, 1,3,4 Pedro Filipe Teixeira, 5 Julia Ring, 6 Julia M. Burkhardt, 7 Nils Burger, 1 Catarina Moreira Pinho, 5 Jelena Tadic, 6 Desiree Loreth, 5,6 Caroline Graff, 10 Friedrich Metzger, 11 Albert Sickmann, 7,15 Oliver Kretz, 8,13 Nils Wiedemann, 1,13 René P. Zahedi, 7 Frank Madeo, 6 Elzbieta Glaser, 5 and Chris Meisinger 1,13,*

1 Institut für Biochemie und Molekularbiologie, ZBMZ, University of Freiburg, 79104 Freiburg, Germany
2 Trinationales Graduiertenkolleg 1478, University of Freiburg, 79104 Freiburg, Germany
3 Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany
4 Spemann Graduate School of Biology and Medicine, University of Freiburg, 79104 Freiburg, Germany
5 Department of Biochemistry and Biophysics, Stockholm University, 10691 Stockholm, Sweden
6 Institute of Molecular Biosciences, University of Graz, 8010 Graz, Austria
7 Leibniz-Institut für Analytische Wissenschaften-ISAS-e.V., 44139 Dortmund, Germany
8 Department of Neuroanatomy, University of Freiburg, 79104 Freiburg, Germany
9 Neurocenter, Department of Neurology, University of Freiburg, 79104 Freiburg, Germany
10 Department of Neurobiology, Care Sciences and Society, Karolinska Institutet-Alzheimer’s Disease Research Center, Karolinska Institutet, 14186 Stockholm, Sweden
11 F. Hoffmann-La Roche Ltd., pRED Pharma Research & Early Development, DTA Neuroscience, 4070 Basel, Switzerland
12 Medizinisches Proteom Center, 44801 Bochum, Germany
13 BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany
14 Co-first author
*Correspondence: chris.meisinger@biochemie.uni-freiburg.de
http://dx.doi.org/10.1016/j.cmet.2014.07.024

SUMMARY

Most mitochondrial proteins possess N-terminal presequences that are required for targeting and import into the organelle. Upon import, presequences are cleaved off by matrix processing peptidases and subsequently degraded by the peptidase Cym1/PreP, which also degrades Amyloid-beta peptides (Aβ). Here we find that impaired turnover of presequence peptides results in feedback inhibition of presequence processing enzymes. Moreover, Aβ inhibits degradation of presequence peptides by PreP, resulting in accumulation of mitochondrial preproteins and processing intermediates. Dysfunctional preprotein maturation leads to rapid protein degradation and an imbalanced organellar proteome. Our findings reveal a general mechanism by which Aβ peptide can induce the multiple diverse mitochondrial dysfunctions accompanying Alzheimer’s disease.

INTRODUCTION

The vast majority of mitochondrial proteins is nuclear-encoded and has to be imported into the organelle from the cytosol. Approximately two-thirds of all mitochondrial preproteins possess N-terminal presequences that direct them to the mitochondrial import machineries (Neupert and Herrmann, 2007; Chacinska et al., 2009; Vogtle et al., 2009). Upon import, presequences are typically cleaved by the mitochondrial processing peptidase MPP in the matrix releasing the mature protein (Haw-litschek et al., 1988; Yang et al., 1991; Vogtle et al., 2009). In several cases, MPP generates import intermediates that are further processed by the octapeptidyl peptidase Oct1/MIP or the intermediate cleaving peptidase Icp55 (Vogtle et al., 2009, 2011; Mossmann et al., 2012; Teixeira and Glaser, 2013). Incomplete processing of mitochondrial preproteins leads to destabilization and accelerated turnover (Yang et al., 1991; Mukhopadhyay et al., 2007; Vogtle et al., 2009, 2011; Varshavsky, 2011). Presequence peptides that have been cleaved by MPP are subsequently degraded by the matrix peptidase Cym1/PreP, a metallopeptidase of the pitrilysin family M16 (Alikhani et al., 2011a). PreP catalyzes turnover of peptides larger than 11 amino acids as well as unstructured proteins (Stahl et al., 2002). Recently, it has been shown that PreP also degrades amyloid-beta (Aβ) peptides that were reported to accumulate in mitochondria of Alzheimer’s disease (AD) patients, and a decline in PreP activity has been observed in AD mitochondria (Manczak et al., 2006; Falkevall et al., 2006; Hansson Petersen et al., 2008; Alikhani et al., 2011b). Aβ import depends on the mitochondrial import machinery (Hansson Petersen et al., 2008). Mitochondrial Aβ appears to affect a multitude of different functions in AD, including respiration, detoxification of reactive oxygen species (ROS), and organellar morphology (Lustbader et al., 2004; Manczak et al., 2006; Yao et al., 2009; Rhein et al., 2009; Morais and De Strooper, 2010; Selfridge et al., 2013). How a single peptide like Aβ can impair all of these diverse, important mitochondrial functions remains elusive.

Here, we investigated the effects of impaired peptide turnover and of Aβ peptide accumulation on mitochondrial functions. We report that mitochondrial preprotein maturation depends on efficient peptide degradation. Moreover, we find that mitochondrial Aβ leads to inhibition of peptide turnover, thereby causing
accumulation of nonprocessed preproteins and processing intermediates within mitochondria. Impaired preprotein maturation modulates protein turnover and changes the global organelar protein composition, which might finally explain the pleiotropic mitochondrial defects observed in AD mitochondria.

RESULTS

Mitochondrial Presequence Processing Depends on Peptide Turnover

In order to analyze the physiological impact of peptide turnover on mitochondrial functions, we characterized a yeast mutant that lacks the PreP homolog Cym1 (Kambacheld et al., 2005). We isolated mitochondria from cym1Δ and wild-type strains and analyzed various mitochondrial proteins by western blotting. Several proteins showed a strong accumulation of their precursors and processing intermediates as well as decreased levels of cleaved, mature proteins in cym1Δ mitochondria in comparison to wild-type (Figure 1A). An antibody raised against the presequence peptide of Sod2 revealed a specific signal of the precursor form in cym1Δ mitochondria, but not in wild-type (Figure 1B). Analysis of cym1Δ mutants that lack critical residues of the metal binding motif (HXXEH) (Table S1, available online; Kambacheld et al., 2005) indicated that accumulation of precursor proteins depends on Cym1 protease activity (Figure 1C).

(continued)
Remarkably, similar diverse mitochondrial dysfunctions have been observed in mitochondria from AD patients and AD mouse models (Morais and De Strooper, 2010; Manczak et al., 2006; Rhein et al., 2009; Alkhani et al., 2011b). These results indicate that physiological consequences of impaired mitochondrial peptide turnover activity correlate with pathological phenotypes observed in AD mitochondria.

To test if presequence processing is affected in the absence of Cym1, we performed in organello imports of radiolabelled preproteins into isolated mitochondria of wild-type and cym1Δ yeast cells. Presequence processing of Sod2 preprotein was impaired in cym1Δ mitochondria, and nonprocessed preprotein accumulated as a Proteinase K-resistant form within the organelle (Figure S1B). Similar defects were observed when import was performed in mas1Δ mitochondria (Figure S1C). The general import efficiency of the presequence import pathway was not compromised in cym1Δ or mas1 Δ mitochondria (tested by the nonprocessed Hsp10 preprotein (Figures S1B and S1C). The processing defect in cym1Δ appeared to be specific for MPP because presequence cleavage of Mprl32 that does not depend on MPP (Nolden et al., 2005) was fully functional in cym1Δ mitochondria (Figure S1D). To directly analyze the dependency of preprotein maturation on peptide turnover, we employed an in vitro processing assay in mitochondrial extracts (Figure S1E) (Falkevall et al., 2006) from wild-type and cym1Δ mitochondria. This allowed the analysis of presequence processing independent of protein import. Presequence peptides were rapidly degraded in wild-type, but not cym1Δ, extracts (Figure S1F). In the presence of a typical presequence peptide (Cox4ΔPreseq) (Yang et al., 1991), the in vitro processing of radiolabelled Sod2 preprotein by MPP was efficiently blocked in the absence of Cym1 (Figure 1E). Cox4 presequence peptides were able to inhibit purified MPP in similar concentrations (Figures S1G and S1H) (Yang et al., 1991). To exclude that the impaired presequence processing activity in cym1Δ is caused by an indirect effect, we added cell-free translated Cym1 protein to the in vitro processing assay. We found that MPP processing activity was restored in cym1Δ extracts in the presence of Cym1 protein, indicating a direct functional link between Cym1 activity and presequence processing (Figure 1F). In addition, overexpression of MPP in the cym1Δ strain, as well as presence of purified MPP in mitochondrial extracts of cym1Δ, was able to suppress the impaired preprotein processing activity (Figures S1I and S1J). We conclude that impaired turnover of presequence peptides leads to inhibition of MPP processing activity. This functional coupling explains the preprotein accumulation observed in cym1Δ mitochondria in vivo (Figures 1A and 1B; Table S3).

Maturation of Precursor Processing Intermediates Depends on Cym1

We wondered why preprotein processing intermediates also accumulated in the cym1Δ mutant (Figure 1A). It has been proposed that PreP/Cym1 requires a minimal substrate length of 11 amino acids (Stahl et al., 2002). However, the intermediate peptidase Ocl1 cleaves off octapeptides (Vögtle et al., 2011). We constructed an ocl1Δcym1Δ double mutant, and the observed synthetic growth defect pointed to a functional link between both enzymes (Figure 2A). Furthermore, we found that processing activity of purified MPP was not affected in the presence of octapeptides (derived from the Oct1 substrate Sdh1; Figure 2B). In contrast, the presence of a presequence peptide efficiently inhibited MPP activity (Figure S1H). However, in vitro processing of the Cox4 precursor that is cleaved sequentially by MPP and Oct1 revealed a specific impairment of the Oct1-dependent processing step in cym1Δ in the presence of octapeptides (Figure 2C). This indicated that Cym1 activity is also affected by accumulation of octapeptides and that an impaired turnover of MPP generated presequences, and Oct1-derived octapeptides lead to inhibition of presequence processing, causing accumulation of preproteins as well as processing intermediates.

To investigate the functional consequences of impaired presequence processing activity, we employed mitochondria from the temperature-sensitive mas1Δ strain that had been shifted to nonpermissive growth temperature for 24 hr to inactivate MPP activity in vivo. Western blot analysis revealed an accumulation of nonprocessed preproteins and reduced levels of mature proteins similar to the phenotype observed in cym1Δ mitochondria (Figure 2D). Thus, inactivation of either peptide turnover (cym1Δ) or presequence processing (mas1Δ) leads to the phenotype of impaired preprotein maturation and reduced amounts of mature proteins (Figures 1A, 1B, and 2D).

Aβ Impairs Mitochondrial Peptide Turnover, Leading to Feedback Inhibition of Presequence Processing

Next, we asked if Aβ peptide that accumulates in mitochondria of AD patients and represents a substrate of human PreP might cause a delay in matrix peptide degradation and thereby induce feedback inhibition of presequence processing enzymes. We found that Aβ peptide was degraded by Cym1 in mitochondrial extracts and by the recombinant enzyme (Figures 3A and 3B). However, degradation of Aβ was slower compared to turnover of presequence peptides (Figure S2A). We then analyzed degradation of Cox4 presequence peptides in wild-type mitochondrial extracts and found that the presence of Aβ, but not of a scrambled form, impaired the peptide turnover capacity of Cym1 (Figure 3C, lanes 5–13 versus 18–26). Scrambled Aβ was not degraded by Cym1 (Figure S2B). We further asked if Aβ can also affect presequence processing activity of MPP. We tested the processing of radiolabelled Sod2 preprotein and observed a striking delay in presequence processing in the presence of Aβ (Figure 3D). We noticed that AβΔ1–40 (unlike the shorter version AβΔ1–28) slightly inhibited activity of purified MPP at higher concentrations (Figures S2C and S2D) and therefore included the shorter version in our functional assays. Taken together, our findings show that Aβ peptide inhibits peptide turnover in mitochondrial extracts, which causes impaired maturation of preproteins.

Mitochondrial Aβ Inhibits Precursor Maturation In Vivo

To analyze the effect of Aβ on precursor maturation in vivo, we attempted to reconstitute the entire mechanism described above in a yeast model that allowed galactose-induced expression of an eGFP-AβΔ1–42 fusion protein harboring a cleavage site for TEV protease (Figure 4A). Coexpression of TEV protease led to release of Aβ peptides from the fusion protein (Figure S3A). To analyze Aβ-induced mitochondrial dysfunctions, we employed the aging-prone strain coa6Δ, which showed a moderate instability of respiratory chain complexes in order to mimick
Aβ Impairs Mitochondrial Preprotein Maturation

Figure 2. Maturation of Precursor Processing Intermediates Depends on Cym1

(A) Synthetic growth defect of cym1Δ oct1Δ double-mutant yeast strain under fermentable conditions (23°C, YPD), [rho0], wild-type strain lacking mitochondrial DNA.

(B) Processing of [35S]F1β precursor by purified MPP is not inhibited by octapeptides. Quantifications represent mean ± SEM (n = 3).

(C) In vitro processing assay of [35S]Cox4 precursor in WT and cym1Δ mitochondrial extracts in the presence of octapeptides. Quantifications represent mean ± SEM (n = 4).

(D) Impaired preprotein maturation leads to imbalanced mitochondrial proteome. Wild-type and temperature-sensitive mas1 strains were grown on YPD medium at 23°C and shifted to nonpermissive temperature (37°C) for 24 hr. Inactivation of the essential MPP subunit Mas1 causes accumulation of precursor proteins that are rapidly degraded. This leads to decreased amounts of mature, fully cleaved proteins (lanes 4–6 and 10–12). In contrast, proteins that do not contain presequences were not affected (lanes 13–18). p, precursor; i, intermediate; c, cleaved protein.

conditions in aged humans (Larsson, 2010; Vögtle et al., 2012), and induced eGFP-Aβ(1–42) expression by growth on galactose. Aβ localized to mitochondria (Figures S3B and S3C) and impaired maturation of Sod2 precursor in soluble mitochondrial extracts (Figures 4B and 4C). The presequence import pathway was not compromised by Aβ (Figure S3D). After induction for 3 days, we observed accumulation of several precursor proteins in mitochondria of the Aβ-expressing strain (Figure 4C), indicating that Aβ inhibits preprotein maturation in vivo. After induction for 5 days, several mitochondrial dysfunctions were observed in mitochondria isolated from the Aβ-expressing strain compared to the control strain. This included increased levels of ROS, a decrease in membrane potential, and impaired oxygen consumption (Figure S3E). Overexpression of Cym1 in the Aβ-expressing strain led to an increased turnover of Aβ, substantial reduction of the accumulating precursors in vivo (Figure S3F), and increased MPP processing activity in vitro (Figure S3G). We conclude that Aβ-mediated inhibition of the peptidase Cym1 leads to an impaired presequence processing activity of MPP, which results in accumulation of preproteins in vivo.

As a further in vivo system, we tested freshly isolated matrix extracts from brain mitochondria of PS2APP mice. This AD model harbors mutations in the PS2 (N141I) and APP (Swedish FAD) genes and shows Aβ accumulation in mitochondria (Figure S3H) (Rhein et al., 2009; Ozmen et al., 2009). Indeed, processing of the Cox4 preprotein was significantly impaired in PS2APP samples compared to that in age-matched wild-type mice (Figure 4D).

Finally, we asked if mitochondrial precursor accumulation caused by Aβ can be observed in AD patients. Therefore, we isolated mitochondria from post mortem brain samples of four AD patients and four age-matched non-AD controls (Table S4). Western blot analysis showed the presence of higher molecular species of the matrix protein MDH2 in all patient samples, but not in controls (Figure 4E). An antibody raised against the presequence peptide of MDH2 that recognizes only the precursor, but not the mature, cleaved protein, confirmed the specific accumulation of the MDH2 preproteins in AD mitochondria (Figure 4F). In order to identify further mitochondrial proteins that might accumulate as preproteins or processing intermediates in AD patients, we screened various antibodies by western blotting and performed a ChaFRADIC analysis (Venne et al., 2013). Several proteins could be identified, including the mitochondrial ribosomal subunit MRPL23 and NDUFA9, a subunit of the respiratory complex I (Figure S3I and Table S5). Taken together, our results show that mitochondrial Aβ causes impaired maturation of preproteins in vivo.
DISCUSSION

So far, mitochondrial presequence processing and peptide turnover have been considered independent reactions. Our findings indicate a functional coupling of both processes. Presequence processing capacity seems to depend directly on efficient peptide clearance in the matrix, as addition of the peptide-degrading enzyme Cym1 to mitochondrial cym1Δ extracts immediately restored MPP presequence processing activity in vitro. Moreover, lack of Cym1 leads to accumulation of immature preproteins in vivo, reflecting the mas1 mutant phenotype with impaired presequence processing activity. Accumulating presequence peptides likely bind to the active site of MPP, thereby competing directly with incoming preproteins (Yang et al., 1991; Taylor et al., 2001). In both cases—mutations in Cym1 and in Mas1—impaired preprotein maturation causes accelerated protein degradation and results in an imbalanced organellar proteome. As a consequence, various mitochondrial functions are affected, e.g., decreased respiration and membrane potential and increased levels of ROS. Interestingly, similar phenotypes have been observed in AD mitochondria (Morais and De Strooper, 2010; Manczak et al., 2006; Rhein et al., 2009; Alikhani et al., 2011b). It is still unclear if mitochondrial dysfunction is the cause or consequence of AD and how mitochondrial damage is connected to other cellular dysfunctions observed in AD (Lin and Beal, 2006; Morais and De Strooper, 2010; Treusch et al., 2011). However, it has been shown that Aβ accumulates in mitochondria of AD patients, where it seems to affect similar diverse functions that we found here in cym1Δ mitochondria. Our results reveal that Aβ causes inhibition of the peptidase PreP/Cym1. In turn, accumulation of presequence peptides leads to impaired maturation and therefore cumulation of mitochondrial preproteins by inhibition of the presequence processing machinery. Indeed, global proteomic studies of AD samples indicated an imbalance of the mitochondrial proteome; presequence-containing mitochondrial proteins in particular were found affected (Rhein et al., 2009; Begcevic et al., 2013).

The results presented here provide a mechanistic model that can explain the diversity of mitochondrial dysfunctions observed in AD (Figure 4G). Herein, the presequence processing machinery plays a central role in the age-dependent vicious cycle of mitochondrial dysfunction in AD. Our findings offer exciting perspectives on the development of diagnostic markers (e.g., to detect preproteins in blood cells of patients that were found to possess mitochondrial dysfunctions; Leuner et al., 2012) and therapeutic strategies (e.g., stimulation of the presequence processing machinery) in AD.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria from Yeast Cells and Mouse and Human Brain Tissue

Mitochondria from S. cerevisiae strains (Table S1), mouse, and human brain tissues (Table S4) were isolated by differential centrifugation. Yeast cells were grown in YPD or YPG medium. Cells were harvested by centrifugation. Wild-type C57Bl/6 mice and transgenic mice (line PS2APP, homozygous for human PS2 N141I and APP Swedish FAD transgenes; Rhein et al., 2009;
Ozmen et al., 2009) of 12 months were sacrificed, and brains were removed. Tissue samples of the temporal cortex of human brains were collected postmortem for diagnostic purposes. Cells or tissues were homogenized by 15–30 strokes in a glass potter. Cell debris and nonbroken cells were removed in two consecutive centrifugation steps (484–1,500 \( \times g \), 4°C). Mitochondria were isolated by centrifugation at 7,000–16,000 \( \times g \) at 4°C. The mitochondrial fractions were aliquoted, snap frozen in liquid nitrogen, and stored at −80°C.

Preprotein Processing and Peptide Degradation Assays in Soluble Mitochondrial Extracts

Isolated mitochondria were washed, reisolated, and suspended in reaction buffer (250 mM sucrose, 10 mM MOPS/KOH [pH 7.2], 80 mM KC1, 1–5 mM MgCl\(_2\), 5 mM KH\(_2\)PO\(_4\)). Mitochondrial extracts were obtained by sonication on ice (five times for 30 s with 30 s breaks; Sonifier250, Branson) or by solubilization in 1% digitonin. Samples were centrifuged at 100,000 \( \times g \) for 45 min or 20,000 \( \times g \) for 10 min at 4°C, respectively. The supernatant was incubated with radiolabelled preproteins, various peptides, and/or chemical amounts of peptidases. Reactions were stopped by the addition of 4\( \times \) Laemmli buffer. Samples were analyzed via SDS-PAGE followed by autoradiography and immunodecoration.

In Vivo Reconstitution of A\( \beta \)-Induced Mitochondrial Dysfunction in Yeast

Yeast strains were transformed with p416TEVcyt and pESC eGFP-A\( \beta \) or pESC ev (empty vector). For selection, cells were grown in selective medium lacking histidine and uracil. Expression of plasmids was induced by shift to selective medium containing 2% galactose. Cells were grown at 30°C for 1, 3, or 5 days. Cell extracts were obtained by post-alkaline extraction, and mitochondria were isolated as described above. Samples were analyzed on SDS-PAGE followed by immunodecoration.

In Vivo MPP Activity Assay

\( \beta \)-MPP (with N-terminal poly-\( \text{His} \) tag) and \( \alpha \)-MPP (Mas1 and Mas2) subunits from \( S. \) cerevisiae were expressed in \( E. \) coli BL21 cells. Cells were grown in
Labeled F1 tion using the rabbit reticulate lysate system (Promega) in the presence of radiolabelled preproteins were synthesized by in vitro transcription/translation using the rabbit reticulate lysate system (Promega) in the presence of 35S-methionine. Chemical amounts of Cym1 (aa 8–989) and control protein were quantified by PhosphorImager scanning. Processing and degradation of the radiolabelled preproteins was monitored by SDS-PAGE and autoradiography.

**Generation of Radiolabelled Precursors and Chemical Amounts of Peptidases**

Radiolabelled preproteins were synthesized by in vitro transcription/translation using the rabbit reticulate lysate system (Promega) in the presence of 35S-methionine. Chemical amounts of Cym1 (aa 8–989) and control protein were quantified by PhosphorImager scanning. Processing and degradation of the radiolabelled preproteins was monitored by SDS-PAGE and autoradiography.

**Statistical Analysis**

Quantified data are shown as mean ± SEM and were obtained from at least three independent experiments.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.07.024.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Drs. M. Yaffe, K. Okamoto, C. Grant, P. Rehling, T. Langer, N. Pfanner, M. Ehrmann, and T. Brummer for strains, plasmids, and antibodies. This work was supported by the NRK 1478, Deutsche Forschungsgemeinschaft (C.M.), Excellence Initiative of the German Federal & State Governments (EXC 294 BIOSS: GSC-4 Spemann Graduate School) (C.M. and N.W.), Baden-Württemberg-Stiftung (F.-N.V.), the Swedish Research Council and the Swedish Alzheimer foundation (E.G.), Fundação para a Ciência e a Tecnologia (EXC 294 BIOSS: GSC-4 Spemann Graduate School) (C.M. and N.W.), Baden-Württemberg-Stiftung (F.-N.V.), the Swedish Research Council and the Swedish Alzheimer foundation (E.G.), Fundação para a Ciência e a Tecnologia (C.M.P.), the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen (J.M.B., A.S., and R.P.Z.), and the FWF for grants (106012) and 7513-C14.

**REFERENCES**


