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Amino acids variations in Amyloid-β peptides, mitochondrial dysfunction, and new therapies for Alzheimer's disease

Hani Atamna

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Abstract Soluble oligomers and/or aggregates of Amyloid-β (Aβ) are viewed by many as the principal cause for neurodegeneration in Alzheimer's disease (AD). However, the mechanism by which Aβ and its aggregates cause neurodegeneration is not clear. The toxicity of Aβ has been attributed to its hydrophobicity. However, many specific mitochondrial cytopathologies e.g., loss of complex IV, loss of iron homeostasis, or oxidative damage cannot be explained by Aβ's hydrophobicity. In order to understand the role of Aβ in these cytopathologies we hypothesized that Aβ impairs specific metabolic pathways. We focused on heme metabolism because it links iron, mitochondria, and Aβ. We generated experimental evidence showing that Aβ alters heme metabolism in neuronal cells. Furthermore, we demonstrated that Aβ binds to and depletes intracellular regulatory heme (forming an Aβ-heme complex), which provides a strong molecular connection between Aβ and heme metabolism. We showed that heme depletion leads to key cytopathologies identical to those seen in AD including loss of iron homeostasis and loss of mitochondrial complex IV. Aβ-heme exhibits a peroxidase-like catalytic activity, which catalytically accelerates oxidative damage. Interestingly, the amino acids sequence of rodent Aβ (roAβ) and human Aβ (huAβ) is identical except for three amino acids within the hydrophilic region, which is also the heme-binding motif that we identified. We found that huAβ, unlike roAβ, binds heme tightly and forms a peroxidase. Although, roAβ and huAβ equally form fibrils and aggregates, rodents do not develop AD-like neuropathology. These findings led us to propose a new mechanism for mitochondrial dysfunction and huAβ's neurotoxicity. This mechanism prompted the development of methylene blue (MB), which increased heme synthesis, complex IV, and mitochondrial function. Thus, MB may delay the onset and progression of AD and serve as a lead to develop novel drugs to treat AD.

Keywords Heme · Rodent amyloid-β · Human amyloid-β · Mitochondria · Peroxidase · Methylene blue · Complex IV

H. Atamna (✉)
Department of Basic Sciences, Neuroscience,
The Commonwealth Medical College,
Tobin Hall, 501 Madison Avenue,
Scranton, PA 18510, USA
e-mail: hatamna@tcmedc.org

Introduction

Overview

Excess amyloid-β (Aβ) peptides ($Aβ_{40}$ and $Aβ_{42}$) in the brain, have been proposed to be the culprits in Alzheimer's disease (AD) (Selkoe 2000). The progressive deterioration in cognitive functions and behavioral skills in AD are likely due to energy hypometabolism, synaptic dysfunction, and oxidative damage, which lead to neurodegeneration (Maurer et al. 2000; Selkoe 2002; Parihar and Brewer 2007). Although these cytopathologies are likely to lead to various types of dementia, their specificity to AD originates from the Aβ peptides whose accumulation is characteristic of this disease. Therefore, understanding the molecular mechanism for AD neurodegeneration requires the elucidation of the biochemistry and cell biology of Aβ. While it is accepted by many scientists that Aβ is the culprit peptide in AD, its connection to mitochondrial dysfunction, energy hypometabolism, synaptic dysfunction, abnormal iron homeostasis, oxidative stress, and memory impairment is still not clear (Walsh and Selkoe 2004; Billings et al. 2005; Golde and Janus 2005; Shankar et al. 2008; Baloyannis 2009).

A decline in complex IV activity (cytochrome *c* oxidase; a major enzyme-complex in the mitochondria) is a hallmark cytopathology in AD brain and occurs in regions most impacted by the neurodegeneration. Interestingly, neuronal activity correlates with the level of complex IV (Wong-Riley 1989). Neurons are specifically susceptible to energy deficiency and impairment in mitochondrial function. Inadequate activity of mitochondria specifically due to the loss of mitochondrial complex IV is likely to result in impairment in redox metabolism, loss of iron homeostasis, oxidative stress, and energy hypometabolism of AD. Energy hypometabolism is one of the most consistent and earliest abnormalities seen in AD and mild cognitive impairment (Mosconi et al. 2008). Decline in energy metabolism appears before the onset of memory deficits (Mucke et al. 2000) and seem to sensitize the neurons to energy deficiency (Arias et al. 2002). The role of insulin in energy deficiency in AD has been discussed before (Atamna and Frey 2007). In this review we discuss the interaction between Aβ and the mitochondria that contributes to mitochondrial dysfunction in AD.

The decline in mitochondrial complex IV in AD has been established (Mutisya et al. 1994; Parker et al. 1994; Maurer et al. 2000). Furthermore, the biochemistry and physiology of mitochondria in the AD brain also appear abnormal. Several studies have also demonstrated aberrations in the Krebs (TCA) cycle in AD brain. Researchers have demonstrated a 30–40% decrease in α-Ketoglutarate dehydrogenase (αKGDH) (Gibson et al. 2000). In addition to the biochemical changes, mitochondria from AD brain exhibit substantial structural changes and abnormalities (Baloyannis 2009). Additionally, the incidence of mtDNA mutations in AD brain exceeds the level expected due to normal aging (Hamblet et al. 2006). The role of excess Aβ in these changes in the mitochondria is not clear.

Amyloid-β peptides and mitochondria

Several studies on autopsy AD brain tissue and transgenic mouse model for AD, localize Aβ inside the cell and within the mitochondria (Knauer et al. 1992; Billings et al. 2005; Manczak et al. 2006; Hansson Petersen et al. 2008; Reddy 2009). A direct link between Aβ and mitochondria was demonstrated as early as 1992. The first localization of AβPP to the mitochondria was seen in the outer membrane of the mitochondria from the brain of AD patients using immunohistochemistry (Knauer et al. 1992). Furthermore, Aβ has been shown to interfere with protein import into the mitochondria (Devi et al. 2006); to bind with Aβ-binding alcohol dehydrogenase (Yan and Stern 2005); and AβPP or its fragments were found in the mitochondria (Crouch et al. 2005). The presence of intracellular and mitochondrial Aβ is consistent with the view that the mechanism of Aβ toxicity may also depend on intracellular and probably intra-mitochondrial factors. This review links Aβ to faulty mitochondrial metabolism in brains of patients with AD.

The prevalent view of Aβ's toxicity states that Aβ aggregates are the culprits that lead to synaptic dysfunction and neuronal demise in AD (Selkoe 2000). Aβ's hydrophobicity in addition to the secondary structures are the most studied features of Aβ peptides and were emphasized as the cause for Aβ neurotoxicity. Aβ oligomers and senile plaques appear to interfere with membranal proteins and structures causing neuronal dysfunction. This mechanism of Aβ's toxicity lacks specificity and does not explain the molecular basis for the key cytopathologies observed in AD such as the selective loss of complex IV, iron homeostasis, AβPP dimerization, and mitochondrial dysfunction. This limitation in current understanding for Aβ's toxicity triggered us and others to search for an alternative mechanisms for Aβ toxicity (Atamna and Frey 2007; Reddy 2009; Wang et al. 2009). Here we discuss key aspects of mitochondrial dysfunction in AD and propose a molecular link between heme metabolism, complex IV, oxidative stress, and the accumulation of Aβ peptides.

Amyloid-β is an unusual neurotoxic agent

Several observations suggest that Aβ are unusual neurotoxic agents. As a result the investigation into their mechanism of action should be unconventional. The gene alteration known to increase Aβ levels in the brain are always dominant or codominant (AβPP and PS mutations, ApoE4 allele), yet at young ages Aβ seems not to harm the brain. Aging is a key risk factor for AD (i.e., Aβ induced-neurotoxicity). These observations suggest that these genes have advantageous metabolic consequences at young ages, which may become deleterious at advanced ages. A different explanation could be that at young ages the metabolism of the brain is capable to cope with the deleterious effects of Aβ (or the products of these genes e.g., ApoE4) but this ability declines at advanced ages. Furthermore, the neuropathological hallmarks of AD (neuritic plaques, neurfibrillary tangles) can be found in brains of cognitively intact older adults. This observation suggests that the formation of neuropathological hallmarks in AD begin years before the onset of the clinical deficits. These findings suggest that the mechanism of Aβ's neurotoxicity is unusual. Aβ seems to interact with cell metabolism in a dynamic way, and the consequences of this interaction depend on the type of the cell, cellular metabolic activity, and age.

Understandings the biochemistry and biology of Aβ

Based on several experimental findings (Atamna et al. 2002; Atamna and Frey 2007) we concluded that Aβ peptide is not metabolically inert. This conclusion lead us to propose a different mechanism for Aβ's toxicity. We pursued the notion that Aβ exerts its toxicity by actively interfering and altering specific components of the cellular metabolism. We searched for a common denominator among the cytopathologies of altered iron metabolism, loss of complex IV, and excess of Aβ. Based on findings from a large number of previous studies and knowledge that the functional form of iron in most metabolic pathways is heme, we suspected that Aβ interferes with heme metabolism. Furthermore, heme-*a* is a unique form of heme that is found only in complex IV. Therefore, we studied the metabolic consequences of excess Aβ on heme metabolism (Atamna and Frey 2007).

We created a heme deficiency in cultured cells to determine if the consequences match the key cytopathologies of AD. We found that the consequences matched and included a selective decline in complex IV, induction of heme synthesis, iron accumulation, mitochondrial dysfunction, increased production of oxidants (e.g. H_2O_2), dimerization of AβPP (suggesting abnormal processing (Scheuermann et al. 2001)), and neuronal cell death following the induction of division or differentiation (Atamna et al. 2001, 2002; Atamna and Boyle 2006b); all are key cytopatholigies of AD. These consequences of heme deficiency were also reproduced by other groups in different experimental systems for heme deficiency (Gatta et al. 2009).

Heme is synthesized in the mitochondria (Dias et al. 2006). Heme (ferroprotoporphyrin IX), the major functional form of iron, is synthesized in all nucleated cells including brain cells by ferrochelatase, which is located in the inner mitochondrial membrane. Ferrochelatase is increased by four-fold in the brains of AD patients while heme is increased approximately three-fold (Venters et al. 1997; Atamna and Frey 2004). Heme, although synthesized in the mitochondrial inner membrane, it can reach all the compartments of the cell (reviewed in (Dias et al. 2006)). Amino acids, peptides, and proteins are believed to transiently bind the newly synthesized heme. The pool of this transiently bound heme is known as "regulatory heme".

The function of regulatory heme is to deliver heme to various metabolic destinations: Complex IV (after conversion to heme-*a*), *b*-cytochromes, *c*-cytochromes, catalases, and oxygenases. In addition, regulatory heme delivers heme to Heme Regulatory Motifs (HRM) in various regulatory proteins. Heme bound to HRM is interchangeable with that present in regulatory heme (Zhang and Guarente 1995). Examples of proteins regulated by heme is RNA-binding protein DiGeorge critical region-8 (DGCR8) (Faller et al. 2007), BTB and CNC homology 1 (Bach1) (Dhakshinamoorthy et al. 2005) and more as described in (Atamna and Frey 2007). These diverse and important roles of regulatory heme in cellular metabolism may account for some of the metabolic consequences of heme deficiency (Atamna et al. 2002; Zhu et al. 2002; Atamna and Boyle 2006a; Chernova et al. 2006).

New mechanism for Aβ's toxicity

We propose the decline in complex IV as a result of heme deficiency is due to lack of availability of heme-*a*. Heme-*a* is essential for the assembly and activity of complex IV, which is the only enzyme that contains heme-*a*. Regulatory heme can be directly used to form cytochromes *b* and *c* with no additional modifications. However, regulatory heme requires biochemical modifications to form heme-*a* through two reactions consecutively catalyzed by two separate enzymes: heme-O synthase (cox10p), which adds a farnesyl group to give heme-O (Mogi et al. 1994), and heme-*a* synthase (cox15p), which oxidizes the methyl group to produce heme-*a* (Brown et al. 2002). We propose that when the level of regulatory heme becomes limiting, the production of heme-*a* declines as compared to heme-*c* or heme-*b* because heme-*a* requires intensive biochemical processing. Under these conditions complex IV declines since the assembly of its 13 subunits (and later its activity) depends on heme-*a*.

The direct link between heme, heme-*a*, and Aβ was provided by the finding that human Aβ binds tightly with heme and heme-*a* to form an Aβ-heme complex (Atamna and Frey 2004; Atamna and Frey 2007). We provided experimental evidence that sequestration and depletion of regulatory heme by human Aβ limits heme availability and causes heme deficiency response in nerve cells including loss of complex IV (Atamna and Frey 2004; Atamna and Boyle 2006a). Thus, heme binding with Aβ leads to the depletion of regulatory heme. Complex-IV-deficient mitochondria are likely to increase the production of superoxide radical and H_2O_2 (Zhao et al. 2003). Interestingly, Aβ-heme exhibits a peroxidase activity, which catalyzes the oxidation of many organic molecules, including serotonin (Atamna and Boyle 2006b) and 4-hydroxyphenylpyruvic acid (the precursor for mitochondrial coenzyme-Q). H_2O_2 produced from the complex-IV-deficient mitochondria, could be used by Aβ-heme peroxidase to enhance the oxidation of cellular and mitochondrial metabolites and proteins (Fawcett et al. 2002; Atamna and Boyle 2006b; Atamna and Frey 2007) (Fig. 1). The decline in complex IV in response to heme deficiency seems to drive the dysfunction of mitochondria, energy hypometabolism, and to explain the increase in oxidative stress. We have

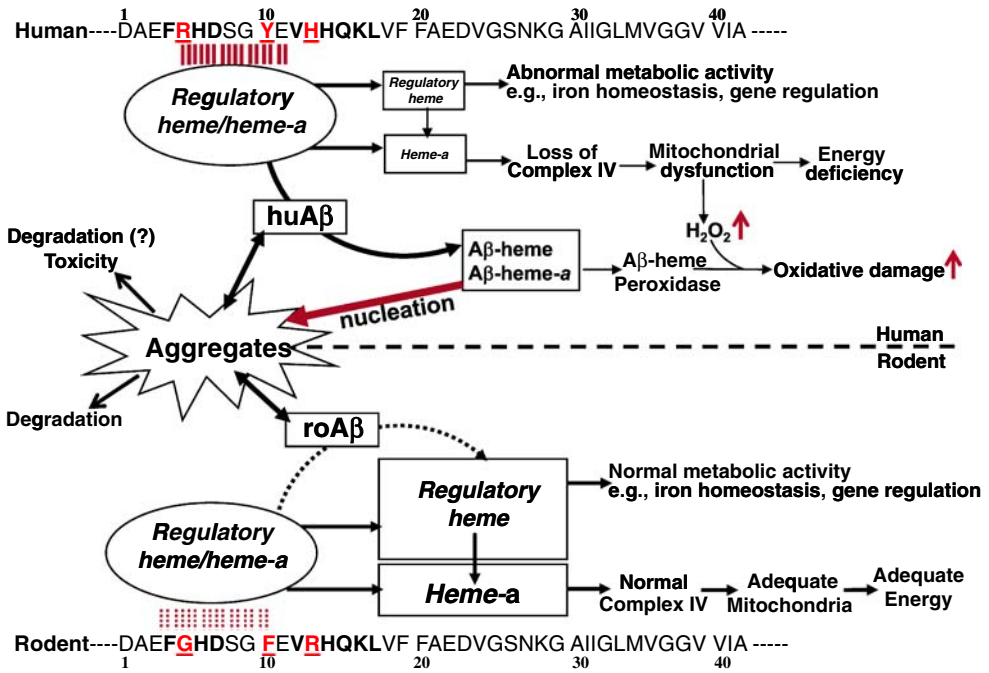

Fig. 1 The amino acid sequence of rodents and human amyloid-β peptides (Aβ) and the proposed outcomes of the differential binding with heme. This scheme depicts the proposed model for huAβ toxicity as compared to roAβ. In addition, the possible effect of huAβ-heme on nucleation of huAβ is also depicted. The *arch-shaped arrows* indicate the efficiency of complexation of heme with the respective Aβ. The *dashed arch-shaped arrow* indicates low efficiency of complexation of heme with roAβ. The *solid-arch-shaped arrow* indicates high efficiency of complexation of heme with huAβ. The *dashed vertical bars* indicate weak binding of heme with roAβ as compared to *solid vertical bars* for huAβ, which indicate tight binding with huAβ. The variable three amino acids in huAβ and roAβ are *underlined*. Amino acids 1-16 are site-H and amino acids 17-42 are site-L (see text for more details)

proposed a molecular mechanism in which excess monomers, dimers, or oligomers of Aβ bind and deplete regulatory heme and form Aβ-heme complexes (Atamna and Frey 2004; Atamna and Boyle 2006a). Because regulatory heme is trapped in Aβ, this could explain why heme synthesis increases in AD (Venters et al. 1997; Atamna and Frey 2004). Thus, the binding of heme by Aβ is a novel and unique feature of Aβ that links abnormal heme metabolism to the key cytopathologies of AD.

Phylogenic variations in Aβ peptides

The amino acid sequence of Aβ can be divided into two main regions: 1) Sixteen amino acids, which make the hydrophilic N-terminal of Aβ (Site-H); and 2) Twenty six amino acids, which make the lipophilic C-terminal (amino acids 17-42 or Site-L) (Fig. 1). We previously proposed that site-H is the main domain to which heme binds (Atamna and Boyle 2006a). Site-H contains the residues Arg, His, and Tyr that are usually found in the heme-binding pockets of the various heme-proteins including peroxidases (Fukuyama et al. 1995). Specifically, one of the three histidine residues in site-H of huAβ is likely to bind and displace the heme-iron out of the plane of the tetrapyrole ring causing the red-shift in the Soret band of heme that we observed when Aβ complexes with heme and heme-*a* (Atamna and Frey 2004; Atamna et al. 2009). Site-L, on the other hand, is responsible for Aβ aggregation and fibrillation (Luhrs et al. 2005).

Interestingly, species that develop AD-like neuropathology have an amino acid sequence of Aβ that is identical to human Aβ (huAβ) (reviewed in (Atamna et al. 2009)) (Fig. 1). Rodents, on the other hand, lack AD-like neuropathology. The amino acid sequence of rodent Aβ (roAβ) is identical to huAβ, except for three amino acids within Site-H (Arg5, Tyr10, His13). The lipophilic site-L is identical among all the species regardless of the presence or absence of AD-like neuropathology (Fig. 1). Mice transgenic for AD overexpress the precursor protein for huAβ, and thus develop some AD-like pathology. Because we propose that heme binding is fundamental for Aβ's toxicity, we hypothesized that roAβ has low affinity for heme compared to huAβ, however it is as efficient in forming aggregates as huAβ.

huAβ and roAβ exhibit differential affinity for heme

We determined and compared the binding constants of roAβ and huAβ with heme. Interestingly, the kinetics

and affinity of heme binding with roAβ and huAβ were remarkably different (Atamna et al. 2009). huAβ binding with heme fits best to biphasic hyperbola with high affinity (K_d=140 nM) while roAβ binding with heme exhibits sigmoidal binding with low affinity (K_d= 1,000 nM).

Close examination of the nonlinear plot of huAβ-heme with heme revealed that huAβ binds with two heme molecules (i.e., biphasic hyperbola). Scatchard transformation exhibits also a concave downward curve (Schwarz 1976); further suggesting either the two heme molecules cooperatively bind with huAβ (or heme binds with the various aggregates of Aβ e.g., dimers, tetramers). Although, the amino acid sequence of huAβ predicts only site-H to bind heme, site-L may also bind with the tetrapyrole macrocycle of heme through hydrophobic interaction. Interestingly, additional binding studies using only site-L revealed that site-L also binds heme (K_d= 210 nM) (Atamna et al. 2009). Scatchard plot of the data of heme binding to site-L is also concave downward, supporting the view that both aggregates and monomers of Aβ could contribute to the complexity of heme binding with Aβ. The macrocycle of heme rather than the heme-iron is likely to participate in heme binding with site-L of huAβ because it lacks amino acids that are known to bind with heme-iron. Therefore, it is not surprising that no changes were observed to the Soret band or peroxidase activity (see below) of heme binding to site-L.

The binding of roAβ with heme, on the other hand, was not as clear or as specific as is the case with huAβ (Atamna et al. 2009). The nonlinear plot for roAβ's interaction with heme best fits a sigmoidal binding curve rather than hyperbola, indicating that roAβ binds with heme, however with low affinity. Scatchard transformation also demonstrated the low affinity binding of roAβ with heme. Only marginal spectral change to heme's Soret band were induced by roAβ, which is likely due to the weak binding between heme and roAβ rather than a result of tight binding. These findings are consistent with the prediction that site-H in roAβ weakly binds with heme.

roAβ also possesses site-L, which was effective in binding heme (K_d=210 nM), yet roAβ binds weakly with heme. This observation led us to propose that the binding of heme to site-L can occur only if heme is tightly bound to site-H. Heme binding to site-H with high affinity may induce conformational changes in huAβ that opens site-L for binding a second heme molecule, which is not the case with heme binding to roAβ.

The binding of two molecules of heme with one molecule of huAβ, suggests that huAβ is very efficient in causing heme deficiency (Atamna and Boyle 2006a).The consequence of heme binding to site-H on the structural changes to site-L (in huAβ) could also be important for triggering the nucleation of Aβ to form large aggregates in vivo (Fig. 1). Furthermore, huAβ-heme may also interfere with huAβ degradation. Thus, the differential binding of heme to site-H in roAβ and huAβ may determine the susceptibility to AD.

Although senile plaques were isolated from AD brains, no heme was found. It is important to emphasize that the conditions used to isolate the senile plaques are known to be inconsistent with the stability of heme or heme proteins. For example, these methods employ a high percentage of formic acid, a high concentration of reducing thiols, and heat (up to 100°C). Furthermore, when histochemical methods were used, the tissue sections were treated with high percentage of hydrogen peroxide during tissue processing to clear any possible endogenous peroxidases. Cleavage of heme macrocycle and the release of iron by hydrogen peroxide or thioles have also been demonstrated (Atamna and Ginsburg 1995), which may explain, in part, the high concentration of iron found in the senile plaques (and probably other metals). Interestingly, using careful histochemical and staining methods demonstrated the colocalization of heme to senile plaques (Cullen et al. 2006). However, the presence of heme as a complex of Aβ in vivo has not been directly demonstrated. Our future research is directed at developing a method to isolate and identify the various types of complexes of Aβ with heme.

huAβ-heme is a peroxidase

The brain of AD patient exhibit higher levels of oxidative damage than those of age-matched normal controls (Nunomura et al. 2001, 2009). The peroxidase activity of huAβ-heme could provide an explanation for the accelerated oxidative damage in AD (Atamna and Boyle 2006a). We compared the effect of huAβ and roAβ on heme's peroxidase activity (Atamna et al. 2009). We found a substantial increase in heme's peroxidase activity only in huAβ-heme. Thus, huAβ-heme may catalytically accelerate oxidative damage in vivo.

The spectral changes to heme are more intense when it complexes with huAβ at sub-stoichiometric concentrations (Atamna et al. 2009). Furthermore, the peroxidase activity increases as the ratio of huAβ to heme increases (Atamna et al. 2009). A huAβ:heme ratio approximately 5:1 seems to form the most efficient catalytic center. Sub-stoichiometric ratios of roAβ to heme, on the other hand, had only a minor effect on the peroxidase activity above the typically minor peroxidase activity of free heme. Consistently, the effect of roAβ on the spectrum of heme was minimal as compared to huAβ, which is consistent with only weak heme binding to site-H in roAβ. Thus, roAβ is unlikely to cause oxidative damage in vivo.

The dependence of the peroxidase activity on the ratio of huAβ to heme is intriguing. One possible explanation is that at low ratios, heme occupies mainly site-H and much less site-L. As the ratio of huAβ to heme increases, the peroxidase activity increases since most of the heme is bound to site-H. Alternatively, the structural changes to huAβ-heme may enhance the peroxidase activity at sub-stoichiometric ratios. These observations suggest that complexation of only a small amount of heme with huAβ is enough to enhance oxidative damage by huAβ in vivo. Although additional heme binding to site-L lowers the peroxidase activity, it exacerbates the degree of heme depletion by huAβ, which accelerates mitochondrial dysfunction and release of oxidants (Atamna et al. 2001, 2002; Atamna and Frey 2004).

huAβ and roAβ exhibit equal tendencies to form fibrils

Site-H varies between huAβ and roAβ while both have an identical site-L. Site-L is key for Aβ aggregation and fibril formation. Thus, we compared the ability of huAβ and roAβ to form fibrils and aggregates. Interestingly, we found that huAβ and roAβ can equally form fibrils as predicted from the sequences of site-L (Atamna et al. 2009). The aggregates of Aβ, which are visually obvious in solutions of both peptides, were further determined by the formation of fibrils using the fluorescence of thioflavin-T (TfT). TfT fluorescence increases upon binding with Aβ fibrils. The formation of fibrils increased as the concentration of Aβ increases regardless of the type of Aβ.

huAβ binds heme tighter than roAβ, does it matter?

AD is neurodegenerative disorder with complex cytopathology that involves various metabolic pathways e.g., AβPP processing, iron homeostasis, mitochondrial electron transport chain, energy homeostasis, neurotransmission, lipid metabolism, and metal homeostasis. Furthermore, aging (and probably environment) are key risk factors in AD. Still an enigma, however, which is the primary pathway to be influenced by Aβ and how?

Interspecies studies and comparative biology can help identify variations in metabolic pathways and expand our understanding of complex biological systems. Studies on less complicated biological systems (e.g., c. elegans, Drosophila) can also increases our understanding of more complex biological systems (i.e., human). The differences and similarities between huAβ and roAβ in heme binding reported here suggest that lack of age-dependent AD-like neuropathology in wild type laboratory mice could, in part, be due to the relatively low affinity that roAβ site-H has for heme. These findings provide support for our hypothesis that tight binding of heme to huAβ in the brains of AD patients depletes regulatory heme and heme-*a*, triggering functional heme-deficiency, loss of complex IV, mitochondrial dysfunction, oxidative stress, and development of the early neuropathologies of AD (Atamna and Frey 2004; Atamna and Boyle 2006a; Atamna and Frey 2007). The catalytic action of Aβ-heme oxidizes cellular metabolites and exacerbates the neurotoxicity of huAβ (Atamna and Frey 2007; Atamna et al. 2009).

The amino acids Arg5, Tyr10, and His13 in site-H of huAβ are replaced in roAβ with Gly5, Phe10, and Arg13, respectively. This phylogenic variation in the amino acid sequence of site-H of Aβ and the differential heme-binding between huAβ and roAβ provides an insight into the mechanism of neurotoxicity of excess Aβ in AD patients. The amino acids Arg, Tyr, and His are known to participate in heme-binding in heme-proteins and peroxidases. Specifically, one of the three histidine residues in site-H of huAβ is likely to bind with and displace the iron atom out of the macrocycle plane of the heme. This displacement for the heme-iron causes a red-shift and enhances the absorbance of the Soret band which we observed when huAβ complexed with heme (Atamna et al. 2009). This provides an explanation for the specific and tight binding of heme to site-H and the creation of huAβ-heme peroxidase. On the other hand, roAβ binds only weakly with heme, which is consistent with the minimal red-shift and minimal enhancement of heme peroxidase activity. These findings stress the significance of the three amino acids: Arg5, Tyr10, and His13 in heme-binding by huAβ.

huAβ is likely to bind with "Regulatory" heme (also known as free heme) in vivo. Regulatory heme provides heme for all the biochemical pathways that depend on heme (e.g., assembly of heme-proteins and gene regulation (Smith 2002; Sassa 2004; Dhakshinamoorthy et al. 2005; Faller et al. 2007)). The intracellular concentration of "regulatory" heme is estimated to be between 30 and 150 nM (Sassa 2004). The K_d of heme binding with huAβ (K_d=140 nM) indicates that excess huAβ is likely to form an Aβ-heme complex in the human brain. Consistent with this conclusion, heme has recently been found to colocalize with the senile plaques in AD brain (Cullen et al. 2006). roAβ, on the other hand, is far less likely to form a stable complex with heme in vivo (K_d=1,000 nM) (Atamna et al. 2009). These findings suggest that depletion of regulatory heme and formation of Aβ-heme contribute to huAβ's neurotoxicity and mitochondrial dysfunction and increase human susceptibility to AD. The phylogenic variations in the amino acid sequence of Aβ result in tight heme-binding to human Aβ which likely contributes to the susceptibility of the human brain to AD. Recent findings implicate intracellular (Tseng et al. 2004; Golde and Janus 2005) and extracellular soluble forms of Aβ in early stages of AD,

rather than large insoluble Aβ aggregates and fibril structures. Both huAβ and roAβ are equally capable of forming fibrils, yet only humans develop AD neuropathology. Our findings suggest that the small aggregates and soluble forms of huAβ (e.g., dimers) are neurotoxic in part because they bind heme through site-H. To the contrary, roAβ exhibits low affinity for heme and it is likely that the complex roAβ-heme does not form in vivo.

Complex IV as a potential therapeutic target for AD

At present, more than 5 million Americans are affected with AD, and the estimated annual health care cost is almost 150 billion dollars. Due to the expected increase in the number of individuals 65 years or older, it is estimated that the total incidence of AD will quadruple by the year 2050 (Brookmeyer et al. 1998). Thus, there is an urgent need to prevent, delay the onset, or slow the progress of AD.

A number of strategies have been proposed for developing therapeutics to treat and prevent AD such as cholinergic and glutaminergic therapies, neurotrophic strategies, anti-Aβ strategies, or therapies targeted at Aβ production or aggregation. Although these approaches are valuable for AD patients, they do not change the underlining disease mechanism. The cholinergic and glutaminergic therapies are available for AD patients, however they only alleviate the symptoms and in most cases are effective for only short period of time.

Mitochondria represent a therapeutic target for preventing various age-related disorders ((Larsson and Luft 1999; Baloyannis 2009; Huang and Hood 2009). By focusing on mitochondrial dysfunction in AD, particularly loss of complex IV, we want to draw the reader's attention to the significance of new research into novel mechanisms of Aβ toxicity. We searched for new strategies to increase the level of complex IV in mitochondria. Our mechanism of Aβ toxicity suggests that if we enhance heme synthesis we may prevent or delay the decline in complex IV. Preventing the decline in complex IV should prevent energy deficiency and free radical production and it may also delay the onset of AD.

We recently showed that Methylene blue (MB) increases heme synthesis, induces complex IV, improves mitochondrial function, increases cell resistance to oxidants, and delays cellular senescence (Atamna et al. 2008). MB is the most effective among the many agents reported to delay cell senescence (Wagner 2006). We extended our in vitro findings to the investigation of the effect of long-term intake of MB in old mice. MB increased the levels of complex IV in brain (by 100%) and heart (by 50%) and restored the age-related declines in cognitive function and muscle strength (Atamna and Gharib, *submitted*). The increase in the activity of complex IV and heme synthesis is intriguing and suggests that MB may be used to prevent the decline in complex IV in AD patients. By inducting of complex IV, MB may increase the cellular energy charge. An increase in energy charge and the metabolic activity of neurons may prevent the decline in learning and memory retention in AD.

High levels of complex IV correlate with increased neuronal metabolic activity (Wong-Riley 1989) and with improved cognitive performance (Luques et al. 2007). MB could increase the brain reserve of complex IV in humans, thus MB could delay or slow the age-related decline in complex IV, preserving mitochondrial function, energy metabolism, and memory retention in AD.

MB is a drug with an extensive medical and safety record in humans; thus, FDA approval for its use in clinical trials in AD patients should be facilitated (discussed in (Atamna et al. 2008)). MB has been in clinical use for about a 100 years to treat a variety of pathological conditions and diseases. One of the most common uses is the chronic treatment of congenital methemoglobinemia, which is due to methemoglobin reductase deficiency. MB is also used to treat methemoglobinemia caused by cyanide, CO, or nitrate poisoning (Clifton and Leikin 2003). Recent clinical uses for MB include preventing the side effects of chemotherapy (e.g., ifosfamide-induced encaphelopathy, and preventing hypotension in septic shock. MB is also used in the treatment of some psychiatric disorders because of its anxiolytic and antidepressant properties. Additional uses for MB include delineation of certain body tissues during surgery and inactivation of infectious agents (reviewed in (Atamna et al. 2008)).

We propose that the reduced form of MB (MBH_2) and MB serve as electron carriers between several dehydrogenases and heme-proteins (e.g., cytochrome *c*) in the mitochondria (Atamna et al. 2008). Complex IV in turn recycles the reduced cytochrome *c*, which triggers the induction of additional complex IV. This mechanism is explained in more detail in (Atamna et al. 2008). We also found an increase in heme synthesis in MB treated cells, which is important since the assembly and activity of complex IV requires adequate production of heme. We believe the increase in heme synthesis is an adaptive response to the increase demand of complex IV. Thus, we suggest that MB can be used to increases brain reserve of complex IV in normal individuals and in patients with mild cognitive impairment, or AD. This increase would help maintain the critical level of complex IV needed for proper mitochondrial function and energy homeostasis.

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