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Down-Regulation of Aminolevulinate Synthase, the Rate-Limiting Enzyme for Heme Biosynthesis in Alzheimer's Disease

Barney E. Dwyer¹, Mark A. Smith², Sandy L. Richardson², George Perry^{2,3}, and Xiongwei Zhu²

¹Research Service, Department of Veterans Affairs Medical Center, White River Junction, Vermont, USA and the Department of Medicine (Neurology), Dartmouth Medical School, Lebanon, New Hampshire, USA

²Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA

³College of Sciences, University of Texas at San Antonio, San Antonio, Texas, USA

Abstract

Heme is an essential cell metabolite, intracellular regulatory molecule, and protein prosthetic group. Given the known alterations in heme metabolism and redox metal distribution and the up regulation of heme oxygenase enzyme in Alzheimer's disease (AD), we hypothesized that heme dyshomeostasis plays a key role in the pathogenesis. To begin testing this hypothesis, we used qRT-PCR to quantify the expression of aminolevulinate synthase (ALAS1) and porphobilinogen deaminase (PBGD), rate-limiting enzymes in the heme biosynthesis pathway. The relative expression of ALAS1 mRNA, the first and rate-limiting enzyme for heme biosynthesis under normal physiological conditions, was significantly ($p < 0.05$) reduced by nearly 90% in AD compared to control. Coordinately, the relative expression of PBGD mRNA, which encodes porphobilinogen deaminase, the third enzyme in the heme synthesis pathway and a secondary rate-limiting enzyme in heme biosynthesis, was also significantly ($p < 0.02$) reduced by nearly 60% in AD brain compared to control and significantly related to apolipoprotein E genotype ($p < 0.005$). In contrast, the relative expression of ALAD mRNA, which encodes aminolevulinate dehydratase, the second and a non-rate-limiting enzyme for heme biosynthesis, was unchanged between the two groups. Taken together, our results suggest regulation of cerebral heme biosynthesis is profoundly altered in AD and may contribute toward disease pathogenesis by affecting cell metabolism as well as iron homeostasis.

Keywords

Alzheimer's disease; heme; neurodegenerative disease

Corresponding authors: Barney E. Dwyer, PhD, Research Service (151), VA Medical Center, 215 North Main Street, White River Junction, Vermont 05009-0001, USA; Tel: 802-295-9363, ex 5478; Fax: 802-296-6308; Barney.E.Dwyer@Dartmouth.edu Mark A. Smith, PhD, Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, Ohio 44106, USA; Tel: 216-368-3670; Fax: 216-368-8964, mark.smith@case.edu.

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INTRODUCTION

Heme, also called iron protoporphyrin IX, is an essential cellular metabolite, regulatory molecule, and protein prosthetic group¹. Because excess “free” heme, which is not bound to protein, is potentially toxic, intracellular heme levels are tightly regulated [41]. As such, disruption of heme homeostasis, whether due to changes in heme biosynthesis, heme bioavailability, or rates of heme degradation, could potentially impair a wide range of cellular functions. In this regard, mounting evidence suggests that cerebral heme metabolism is perturbed in Alzheimer’s disease (AD). For example, increased heme oxygenase-1 (HO-1) levels found in the brains of AD patients suggests increased heme degradation rates [16,30, 35,36,40]. In addition, amyloid- β (A β), the principle protein component of Alzheimer plaques, is increased in AD and binds heme with possible deleterious consequences, including development of functional heme deficiency and impaired assembly of mitochondrial cytochrome c oxidase (respiratory complex IV) [4]. Acquisition of peroxidase activity by heme-A β complexes may increase oxidative stress [3], but a protective effect of heme-A β complexes cannot be ruled out. Thus, others have shown a protective role for A β , which can bind iron and serve a chelator/antioxidant role in the disease [18,19,33,38].

The biosynthesis of heme requires eight enzymes located in both mitochondria and cytoplasm [1]. The first enzyme in the pathway, aminolevulinate synthase (ALAS, EC 2.3.1.37), catalyzes the condensation reaction of glycine and succinyl-CoA to form 5-aminolevulinic acid (ALA). This condensation reaction takes place in mitochondria, and is the rate-limiting reaction in heme biosynthesis under most conditions. The next two reactions occur in the cytoplasm. Aminolevulinate dehydratase (ALAD, EC. 4.2.1.24) catalyzes the condensation of two molecules of ALA to form the monopyrrole porphobilinogen. Porphobilinogen deaminase (PBGD, EC 4.3.1.8) catalyzes the assembly of four molecules of porphobilinogen into the linear tetrapyrrole hydroxymethylbilane. PBGD becomes rate limiting for heme biosynthesis when ALAS is bypassed, such as when ALA is administered during photodynamic therapy for cancer [9], or when heme demand is increased in individuals with the genetic trait for acute intermittent porphyria [2]. Under normal physiological conditions, cellular heme deficiency caused by increased heme demand increases ALAS activity by both stabilizing ALAS1 mRNA and relieving heme-mediated inhibition of mitochondrial import of newly translated ALAS [23]. We reasoned if heme deficiency developed in AD brain, ALAS1 mRNA would be increased through either increased expression or increased half-life. However, in contrast, we found decreased expression of both ALAS1 and PBGD in AD brain compared to aged-matched controls. This observation supports the hypothesis that heme biosynthesis is altered in AD brain, and raises the prospect that heme levels are increased in AD brain in spite of increased heme oxygenase.

MATERIALS AND METHODS

Frozen frontal cortical tissue from clinically and pathologically confirmed cases of AD (ages 65-85, mean 76.3) and non-AD age-matched controls (ages 65-83, mean 73.4) using National Institute of Aging (NIA) and Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) criteria was obtained at autopsy by the Case Western Reserve University Brain Bank under an IRB-approved protocol. Table 1 lists individual case information.

¹Several forms of heme are present in mammalian cells [22] C.T. Moraes, F. Diaz, A. Barrientos, Defects in the biosynthesis of mitochondrial heme c and heme a in yeast and mammals, *Biochim. Biophys. Acta* 1659 (2004) 153-159. Heme-*b* is the prosthetic group of most hemoproteins including *b*-type respiratory cytochromes, the cytochrome P450 enzymes, and hemoglobin. Heme-*a*, which is the heme prosthetic group of cytochrome c oxidase (respiratory complex IV), its sole known function in mammalian cells, is an enzymatically modified form of heme-*b*. Heme-*c* is present in c-type cytochromes. In the present study, the terms heme and heme-*b* are used interchangeably. Other forms of heme are referred to specifically.

RNA extraction and RT-PCR

Total RNA was extracted from frozen brain tissue, using the RNeasy Mini Kit (Qiagen), following the instructions of the manufacturer. RNA was treated with DNase (Turbo DNase, Ambion) to remove trace DNA. RNA was converted to cDNA using the RETROScript RT-PCR kit (Ambion) according to the manufacturer's instructions. Briefly, the purified total RNA (100 ng) was reverse transcribed with cloned Moloney murine leukemia virus reverse transcriptase (5 U) by incubating at 44°C for 60 min, followed by heating at 92°C for 10 min. The resulting single-strand cDNA was then amplified using a pair of primers for each target gene (925 pmol) and Taq DNA polymerase (2.5 U) in a DNA thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler). An initial denaturation step (95°C for 5 min) was followed by 27 cycles (95°C for 45 s, 56°C for 45 s, 72°C for 45 s) and a final extension period (72°C for 5 min). The RT-PCR products were then subjected to 1.5% agarose gel electrophoresis. DNA in each sample was quantified using a UVP imaging system. Gene expression was relatively quantified from the integrated optical density of the PCR product.

Primer sequences for each gene were as follows:

Aminolevulinate synthase (ALAS, EC 2.3.1.37):

5'-CAA AAC TGC CCC AAG ATG AT-3' (forward)

5'-CTG TTG GAC CTT GGC CTT AG-3' (reverse)

Aminolevulinate dehydratase (ALAD, EC 4.2.1.24):

5'-GAG CCC TTA GGG AGG CAG-3' (forward)

5'-CCG AAG TAG TGG GTG GAA GT-3' (reverse)

β -actin:

5'-CAT GTA CGT TGC TAT CCA GGC-3' (forward)

5'-CTC CTT AAT GTC ACG CAC GAT-3' (reverse)

The design of primers pairs distinguishes between erythroid and non-erythroid expression of the heme biosynthetic enzymes [1]. The ubiquitous or "housekeeping" form of ALAS1 is located on chromosome 3. The ALAS1 forward primer targets exon 1A of ALAS1 and would be expected to detect the major form of ALAS1 and a minor form of ALAS1, if present, which lacks exon 1B [31,32]. The erythroid-specific form of ALAS (ALAS2) is encoded by a gene on the X chromosome, and is undetected by the primer pairs selected. ALAD is encoded by one gene located on chromosome 9 that has two alternatively spliced, non-coding exons that lie above a common transcriptional start site. The ALAD forward primer targets exon 1A, which is present only in the "housekeeping" form of ALAD.

Real time RT-PCR for PBGD

Real time RT-PCR was used to quantify relative PBGD expression normalized to β -actin. TaqMan PCR assay for PBGD was performed in triplicate on cDNA samples in 96-well optical plates on an ABI StepOne Plus Thermocycler (Applied Biosystems). For each 20 μ l TaqMan reaction, 2 μ l cDNA was mixed with 10 μ l 2 $\times$ TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 1 μ l of 20 $\times$ TaqMan assay mix. Standard fast PCR parameters were used. Data is expressed as relative quantity (RQ value).

A single gene on chromosome 11 encodes PBGD. The PBGD forward primer targets the splice junction between exons 1 and 3. In erythroid tissue, an erythroid specific promoter that lies upstream of exon 2 regulates PBGD transcription. Exon 2 and 3 are spliced and exon 1 is lost.

Data analysis

The experiments were performed in triplicate and data is expressed as the relative levels of AD samples compared to controls. Statistical analysis was performed using the student's t-test.

RESULTS

Using RT-PCR, we found that the relative expression of ALAS1 mRNA was reduced about 90% in AD brain (n=5) compared to controls (n=5) ($p < 0.05$) (Figure 1). Similarly, the relative expression of PBGD mRNA measured by real time RT-PCR was reduced about 60% in AD brain (n=13) compared to control (n=8) ($p < 0.02$) (Figure 2). On the other hand, the relative expression of ALAD mRNA in AD brain and that of β -actin mRNA (the reference transcript), showed no significant changes from control (Figure 1). No correlations were found within the AD group between mRNA expression and age or postmortem interval. Among the AD cases, PBGD mRNA levels were significantly ($p < 0.05$) lower in APOE 4/4 compared to APOE 3/3 cases (Figure 3).

DISCUSSION

In this study, we show significant reductions of the rate-limiting enzymes involved in heme biosynthesis, ALAS1 and PBGD, in the postmortem cortex of AD subjects, providing additional evidence of abnormal heme homeostasis in AD. Knowledge of cerebral heme status is potentially important for understanding the pathophysiology of AD. Pharmacologically induced heme deficiency causes senescence-associated changes in cultured cortical neurons, including neuritic degeneration [5]. Inhibition of heme biosynthesis in nerve growth factor-induced PC12 cells inactivated pro-survival Ras-ERK 1/2 signaling, activated JNK signaling, and is proapoptotic [37]. Thus, inhibition of heme biosynthesis could contribute to the altered levels of activated ERK and JNK seen in neurons in AD [29,42,45,48]. In addition, in HEK cells over expressing human amyloid precursor protein (APP₆₉₅), suppression of heme biosynthesis by inhibition of mitochondrial ferrochelatase, with small interfering RNA and with N-methylprotoporphyrin IX, severely perturbed mitochondrial energy metabolism and altered APP processing [10]. Thus, inhibition of heme biosynthesis could also contribute to mitochondrial dysfunction and energy perturbations seen in neurons in AD [27,28].

ALAS1

The ALAS1 gene promoter is rich in regulatory elements. Thus, regulatory pathways such as insulin-mediated transcriptional inhibition of ALAS1 [14] could be relevant in AD where insulin signaling abnormalities exist [7]. However, a possible explanation for decreased ALAS1 mRNA expression is the observation that cellular heme-*b* was significantly greater in temporal lobe of AD subjects [4]. Thus, elevated heme-*b* destabilizes ALAS1 mRNA [23] and possibly suppresses ALAS1 mRNA expression, although the latter mechanism is controversial [17]. A key question is why heme excess would develop in AD. Total HO activity in normal brain (mostly HO-2) is high and comparable to that in spleen, an organ actively involved in degradation of senescent red blood cells and hemoglobin disposition [20]. Increased HO-1 in AD brain is well documented [16,30,35,36,40]. Increased HO-1 in AD is expected because oxidative stress is prominent [24,25,39] but increased HO-1 is also consistent with excessive heme accumulation because heme is an inducer of HO-1. Increased bilirubin in CSF of AD cases suggests that HO activity is increased [16]. Nevertheless, interaction of HO with amyloid precursor protein [41] or reduced availability of NADPH, a substrate for heme-degradation by HO [40] could partially suppress HO enzymatic activity in AD. Further study will be needed to confirm whether heme-mediated down regulation of ALAS1 mRNA expression is important in AD, and whether ALAS1 mRNA might be a useful biomarker for cerebral heme levels.

PBGD

In the brains of AD patients, PBGD mRNA levels were found to be less than half that in controls. We cannot conclude from the present data that regulation of ALAS1 and PBGD mRNA is coordinated, and there is no evidence for regulation of PBGD mRNA by heme levels in mammalian cells. Unique disease-related factors, such as APOE genotype, could be important as suggested in Table 1. In addition, the housekeeping promoter of rat PBGD contains several putative regulatory motifs including SP1 [46], a transcription factor which is abnormal in AD [34]. Decreased expression of PBGD mRNA in AD may be unrelated to heme biosynthesis. A “moonlighting” function for PBGD in the cell nucleus, which in glioma cells may be linked to tumorigenesis and cell cycle activity, has been reported [11,12]. Moreover, 50% reduction in PBGD mRNA expression by itself may not significantly affect cerebral heme synthesis. An inherited deficiency in PBGD causes acute intermittent porphyria, the most common cause of acute hepatic porphyria [1,2]. In spite of a 50% reduction in PBGD enzymatic activity in latent individuals, symptoms of an “acute” porphyric attack do not develop unless hepatic heme demand is increased by administration of porphyrinogenic drugs such as barbiturates [2]. Such a scenario is reminiscent of the Two-Hit Hypothesis for AD positing that neuronal dysfunction is elicited by a first hit (such as development of cellular oxidative stress) which compromises the ability to deal effectively with a second unrelated hit [47,49]. PBGD mRNA is a frequently used control message for gene expression measurements by quantitative RT-PCR [6]. Our data underscore the need for validation if PBGD mRNA expression is used for that purpose.

ALAD

In contrast to ALAS1 and PBGD, ALAD mRNA levels were unchanged in AD brain. Under normal conditions, cellular ALAD is present in great excess and is unlikely to become rate limiting for heme biosynthesis; for this reason Doss porphyria, a condition marked by ALAD deficiency, is the rarest form of porphyria [1,2,21]. Cellular ALAD may be present in excess of requirements for heme biosynthesis because it “moonlights” as a component of the 26S proteasome [13]. The observation that ALAD mRNA is unchanged in control and AD brain, even though proteasomal abnormalities are reported in AD [26], suggests that ALAD could be potentially useful as an endogenous, unregulated reference gene for gene quantitative RT-PCR in AD and other neurodegenerative diseases.

In summary, increased HO-1 in AD suggests heme turnover is altered in AD [16,30,35,36, 40]. Heme-binding to fibrillar A β [4] suggests intracellular heme distribution is altered in AD, and raises the prospect that functional cellular heme deficiency may develop. Mitochondrial abnormalities in AD [8,15,44] raise the possibility heme biosynthesis is impaired because heme biosynthesis occurs both in cytoplasm and in mitochondria and thus requires mitochondrial integrity. The present study provides evidence that heme biosynthesis is altered in AD. While the significance of altered heme metabolism in AD pathogenesis remains to be elucidated, it is likely to be important given the wide range of cellular functions that require heme [43].

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Abbreviations

A β , amyloid- β peptide; AD, Alzheimer's disease; ALA, 5-aminolevulinic acid; ALAS, aminolevulinate synthase; ALAD, aminolevulinic acid dehydratase; PBGD, porphobilinogen deaminase (also referred to in literature as hydroxymethylbilane synthase).

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Figure 1. Semi-quantitative analysis of mRNA in human AD and control brains. ALAS is dramatically reduced in AD brains. ALAD and Actin (housekeeping gene) show no change in relative mRNA levels (* $p < 0.05$).

Figure 2.

Real time RT-PCR analysis of PBGD in human brain tissue of AD cases compared to controls. PBGD mRNA is reduced in AD brains by about 60% (* $p < 0.02$).

Figure 3.

Real time RT-PCR analysis of PBGD in human brain tissue of AD cases correlated with APOE genotype. Expression of PBGD mRNA was significantly lower in cases with the APOE 4/4 genotype compared to those with the APOE 3/3 genotype (* $p < 0.005$).

Table 1

List of cases used for real time RT-PCR analysis of PBGD. There was no significant difference in age or postmortem interval (PMI) between control and AD groups. A subset of these cases was used for ALAS and ALAD analysis (*).

Alzheimer Disease (n=13)				
Age	PMI (hrs)	Sex	APOE	Braak Stage
65	3	F	3/3	V-VI
69	8	M	3/3	V-VI
69	6	F	4/4	V-VI
72*	4	M	3/4	III-IV
72*	5	M	3/3	V-VI
77	4	F	3/3	V-VI
78	3	M	4/4	V-VI
79	2.5	F	3/4	V-VI
80	4	F	4/4	V-VI
80*	5.5	M	3/4	III-IV
83*	8	M	3/4	III-IV
83*	3	F	3/4	V-VI
85	6	F	3/4	V-VI
Control (n=8)				
Age	PMI (hrs)	Sex	APOE	Braak Stage
65*	14	M	3/3	0
65	2	M	3/3	0
65	1	F	3/3	0
74*	8	M	2/4	I-II
75*	17	F	3/3	0
79*	2	F	3/3	I-II
81*	27	M	2/3	0
83	6	M	3/3	Lewy body dementia