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The Herp Protein Pathway is Not Involved in the Pro-Amyloidogenic Effect of Hyperhomocysteinemia

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Abstract

Diet-induced high circulating levels of homocysteine, also known as hyper-homocysteinemia (HHcy), is associated with an acceleration of Alzheimer's disease-like amyloidosis. Herp is a homocysteine-responsive stress protein, which has been shown to increase the formation of amyloid- β ($A\beta$) via interaction with presenilins *in vitro*. The aim of our paper was to investigate the functional role that Herp plays in HHcy-induced amyloidosis. Amyloidosis secondary to diet-induced HHcy in Tg2576 mice is associated with an increase of Herp protein and mRNA levels. By contrast, no other stress-related proteins are altered by the same diet regimen. Compared to wild type animals, brains from a genetically induced HHcy mouse model did not manifest any significant change in Herp levels. Cells stably over-expressing human $A\beta$ PP Swedish mutant incubated with high levels of homocysteine had an increase in $A\beta$ formation, but no change in Herp level. Finally, over-expression of Herp did not result in any significant modification of $A\beta$ levels. We conclude that the Herp protein pathway is unlikely to be directly involved in the pro-amyloidotic effect of HHcy.

Keywords

Amyloid- β ; amyloid- β metabolism; endoplasmatic reticulum stress; Tg2576; Tg-278Cbs^{-/-}

INTRODUCTION

Homocysteine is a thiol-amino acid produced in the methionine cycle, and in humans its plasma concentrations are normally kept low by folate and enzymes such as cystathionine-beta-synthase (CBS) [1]. Elevated plasma concentration of homocysteine, also known as hyperhomocysteinemia (HHcy), has been widely recognized as a risk factor for cardiovascular diseases including atherosclerosis and coronary heart disease [2–4]. Recent studies found that HHcy is also associated with an increased risk of Alzheimer's disease (AD) onset [5–7]. Hence, understanding the link between HHcy and AD is necessary to develop meaningful therapeutic strategy to treat people bearing this risk factor.

However, the possible molecular mechanism(s) involved in the association between HHcy and AD remain uncertain. Homocysteine can alter DNA methylation [8] and impair DNA repair resulting in increased accumulation of DNA damage and trigger apoptosis [9]. It can also promote oxidative stress [10,11] and activate glutamate receptors thereby rendering

neurons vulnerable to excitotoxicity [12]. Interestingly, there is evidence from *in vitro* and *in vivo* studies showing that HHcy can also increase amyloid- β (A β) formation, which could then aggregate and eventually deposit as amyloid plaques in AD [8,13,14].

Previous studies have shown that homocysteine induces an endoplasmic reticulum (ER) stress response and leads to the expression of a novel protein, called homocysteine-induced endoplasmic reticulum protein (Herp) [15]. Herp is a membrane-associated ER protein which is involved in ER-associated degradation processes [16,17]. Immunohistochemical analysis of AD brain found that Herp localizes in activated microglia around the senile plaques areas [18]. Moreover, its over-expression in cells was reported to increase A β production, probably through a γ -secretase-dependent pathway as Herp putatively binds to some components of γ -secretase [18,19]. However, no data are available to evaluate the effect of Herp on A β production *in vivo*.

In the present study, we investigated the role of Herp in modulating A β formation by using different *in vivo* and *in vitro* models of HHcy. Our results demonstrate that Herp is unlikely to be directly involved in the pro-amyloidogenic effect of HHcy.

METHOD AND MATERIALS

Tg2576 mice and diet treatments

All animal procedures were approved by the Institutional Animal Care and Usage Committee, and in accordance with the National Institute of Health guidelines. Tg2576 transgenic female mice expressing hA β PP with the Swedish mutation (K670N/M671L) [20] were used in the diet-induced HHcy model. All mice were genotyped by polymerase chain reaction (PCR) analysis using tail DNA and were kept in a pathogen-free environment, on a 12-hour light/dark cycle and had access to food and water *ad libitum*.

All the animals used in the present study have already been described in details in another paper [21]. Briefly, starting at 8 months of age, mice were randomized to two different diets: standard rodent chow enriched in methionine (7.7 g/Kg) ($n = 6$) or vehicle ($n = 7$) [21]. Diets were custom-made, prepared by a commercial vendor (Harlan Teklad, Madison, WI), and matched for kilocalories [22]. Mice were sacrificed after 7 months on the diets at an age of 15 months. After sacrifice, animals were perfused intra-cardially with ice-cold 0.9% PBS containing 10 mM EDTA. Brain was then removed and dissected, cortex homogenates were extracted in RIPA buffer for biochemistry analyses, as previously described [23].

Tg-278 *Cbs*^{-/-} mice

Tg-278 *Cbs*^{-/-} mice, whose plasma homocysteine level reaches more than 200 μ M, were generated on a C57B6 background as described before [24]. Three 12-month-old Tg-278 *Cbs*^{-/-} and four age-matched C57B6 mice were sacrificed and their brains harvested for biochemical analyses as described above.

Western blot analyses

RIPA fraction of brain homogenates were used for western blot analyses. Samples were electrophoresed on pre-casted gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to nitrocellulose membranes. Antibodies and dilutions used for western blots were: anti-A β PP N-terminal raised against amino acids 66–81 for total A β PP (22C11; 1:500; Chemicon International, Temecula, CA, USA), anti-Herp (1:500; Proteintech Group, Chicago, IL, USA), anti-BIP (1:500; Abcam Inc., Cambridge, MA, USA), anti Hsp90 (1:500; Assay Designs, Inc, Ann Arbor, MI, USA) and anti- β -actin (1:4000; Santa Cruz

Biotechnologies, Santa Cruz, CA, USA) were used. Odyssey infrared imager system (Li-COR, Lincoln, NE, USA) was used for detection.

Real-time quantitative RT-PCR amplification

RNA isolation from mice brain was performed with RNeasy mini Kit (Qiagen Inc. USA, Valencia, CA, USA) and reverse transcription was performed using RT² first strand kit (SABiosciences, Frederick, MD, USA). RT-PCR tests were performed using RT² qPCR primer assays (SABiosciences, Frederick, MD, USA) with Roche Lightercycler system (Roche Applied Science, Indianapolis, IN, USA). PCR-primer of Herp (PPM27469A) and β -actin (PPM02945A) were also purchased from SABiosciences (SABiosciences, Frederick, MD, USA). All the procedures were conducted following the company provided protocols.

In vitro cell studies

CHO-A β PPsw cells stably expressing human A β PP Swedish mutation were previously described [25]. Cells were maintained in McCoy's Medium (supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin) containing 200 μ g/ml G418, and treated with 500 μ M DL-homocysteine (Fluka Chemical, Milwaukee, WI, USA) for 4 days. On the third day, cell media were changed and fresh DL-homocysteine added. Cell lysate were then collected in RIPA buffer for western blot analyses [21].

HEK293-A β PP cells which stably expressing wild type human A β PP and CHO-APPsw cells were used for Herp transfection studies. Herp pcDNA3.1 plasmid and empty pcDNA3.1 were kind gifts from Dr. Linda M. Hendershot (St. Jude Children's Research Hospital, Memphis, TN USA). Transfection was performed with Lipofectamine 2000 (Invitrogen Carlsbad, CA, USA) following standard protocol. After 48 h, conditioned media were collected for A β quantification and cell lysate for western blot analyses.

Human A β ₁₋₄₀ and A β ₁₋₄₂ levels in the cell medium were assayed by sensitive sandwich ELISA kits (IBL America, Minneapolis, MN, USA).

All the *in vitro* experiments were repeated at least 3 times in duplicates.

Data analysis

Data analyses were performed using SigmaStat for Windows version 3.00. Statistical comparisons between the different treatment groups were performed by student t-test. Values in all figures represent mean $\pm$ S.E.

RESULTS

Previously, we reported that 7-month regimen on a methionine-rich diet results in HHcy (plasma Hcy around 30 μ M) which is associated with an increases A β levels and deposition in the brains of Tg2576, a model of AD-like amyloidosis [21]. In the current study, we focused on the role that Herp might play in the HHcy-induced A β elevation.

First, we compared protein level of Herp in the Tg2576 mice on control diet (Ctrl group) with the methionine-rich diet (Met group). As shown in Fig. 1, we found that brain homogenates from the diet-induced HHcy group (Met group) had significantly higher level of Herp protein than mice fed with regular chow (Ctrl group). This increase at the protein levels was also associated with a similar pattern when we examined Herp mRNA level by quantitative RT-PCR (Fig. 1C). However, no statistically significant correlation between Hcy and Herp levels was detected.

Since Herp is an ER stress response protein, we next investigated whether other stress proteins were altered in the Met group. To this end, we found that levels of two other ER stress-induced proteins, Bip/GRP78 and heat shock protein 90 (Hsp90) (Fig. 1), were unchanged in the Met group when compared to control animals.

To test the hypothesis that the Herp increase in the Met group is secondary to the significant increase in circulating levels of homocysteine, we analyzed Herp protein levels in a model of genetically-induced HHcy, Tg-278 *Cbs*^{-/-} mouse. This mouse model lacks the enzyme CBS that is necessary to break down homocysteine and spontaneously develops HHcy (plasma homocysteine level > 200 μ M) [24]. Compared with wild type littermates, brains from Tg-278 *Cbs*^{-/-} mice had no significant difference in Herp protein and mRNA levels (Fig. 2). Similar results were obtained with another stress-related protein Bip and Hsp90 (Fig. 2).

Next, we studied the behavior of Herp protein in an *in vitro* model of HHcy. Confirming previous studies we observed that incubation of 500 μ M homocysteine with CHO-APPsw cells results in a significant increase in A β ₄₂ levels in the medium ($131.6 \pm 7.5\%$, over controls; $p < 0.05$) [21]. In this *in vitro* model, however, the high levels of homocysteine failed to alter Herp protein levels (Fig. 3).

Finally, the effect of Herp on A β production was directly tested by over-expressing this protein in CHO-A β PPsw cells. After transfection, Herp levels were significantly increased in the Herp-plasmid transfected cells compared to vehicle treated ones (Fig. 4). However, over-expression of Herp did not significantly influence the amount of A β released in the conditioned media, or the levels of the A β precursor protein (APP) (Fig. 4). These results were confirmed also by transfecting Herp into HEK293-A β PP cells. As shown in Fig. 5, the expression of higher levels of Herp did not result in any significant difference in A β levels or A β 3PP when compared with empty vector controls.

DISCUSSION

Since HHcy was recognized as a risk factor of AD, many efforts have been made to understand how this factor may affect the development of AD neuropathology. One possible mechanism is that HHcy could increase the A β formation, thus accelerate the disease development [13,14]. Recently, our laboratory provided evidence supporting this hypothesis and suggested that the GSK3 signaling pathway, by modulating the γ -secretase complex activity, might be involved in HHcy-induced A β elevation [21].

However, since *in vitro* homocysteine can induce Herp, which in turn by binding to γ -secretase induces an increase in A β formation [18,19], in this paper we hypothesized that Herp could also be involved in the HHcy-induced A β elevation we observed *in vivo*.

To the best of our knowledge, the present study provides the first piece of evidence showing that diet-induced HHcy, which results in increase A β levels and deposition [21], is associated with an up-regulation of both Herp protein and mRNA levels *in vivo*. By contrast, other ER stress response proteins, Hsp90 and Bip, do not show any changes in the diet-induced HHcy group. It is possible that this finding is secondary to the fact that Herp up-regulation is more sensitive to ER stress than Bip or HSP90 [15]. Recent data suggest Herp may be also involved in proteasome-mediated degradation and neural survival. For example, over-expression of Herp was reported to promote survival of neural cells and reduce ER Ca²⁺ release under ER stress [16]. These functions may be also related with the elevated Herp level observed in Met group in our study.

However, in a genetically induced HHcy mouse model, Tg-278 *Cbs*^{-/-}, neither Herp protein nor mRNA level showed any significant changes. This discrepancy may due to the difference between these two HHcy models. For example, compared with the diet-induced HHcy, the genetically-induced HHcy model manifests plasma homocysteine levels much higher (Tg-278 *Cbs*^{-/-} mice: 250 μ M vs. diet-treated Tg2576: 30 μ M), and HHcy starts at a very early stage [21,24]. Since a previous paper found that Herp protein is up-regulated by low level of ER-stress, but decreased in extreme high ER-stress conditions [16], it is possible that this is the case for the Tg-278 *Cbs*^{-/-} mice.

To further investigate the relationship between HHcy, Herp, and A β , next we moved to an *in vitro* model. Confirming our previous studies, we observed that incubation of CHO-A β PPswe cells with high levels of homocysteine resulted in a significant increase in A β formation [21]. However, under these conditions HHcy failed to induce any significant change in Herp levels, suggesting that the elevated A β level observed in this *in vitro* model is unlikely to be related with Herp.

Previous studies reported that *in vitro* over-expression of Herp results in a significant increase in A β formation [18,19]. By contrast, in our hands, increased expression of Herp protein levels failed to elevate A β levels in two cell types. These different results may be due to the fact that previous reports used stable transfection instead of transient transfection as we did. However, the failure of an effect of transient Herp transfection on A β formation indicates that the influence of this protein on this phenomenon is probably weak and unlikely to be a predominant factor in the HHcy-induced A β elevation *in vivo*.

Herp protein was discovered only a decade ago [15]. Our understating of its function and working mechanism is still very limited. Since the relationship between Herp protein level and its activity is not well established yet, we cannot rule out the possibility that while Herp protein level remains the same, its activity is actually increased. Future studies using neuronal primary culture, Herp knockout cells, or animal model may provide us with more conclusive results.

In summary, although Herp protein and mRNA levels up-regulation are associated with a dietary model of HHcy and amyloidosis, our study fails to provide any evidence in support of the hypothesis that this protein might directly influence A β levels. Thus, Herp was not associated with an A β elevation by either homocysteine incubation, or in an Herp protein over-expressing system. Therefore, we conclude that the increase in the Herp pathway we observed in the Tg2576 mice brains is probably secondary to the HHcy-induced proamyloidotic effect.

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Fig. 1. Herp protein and mRNA levels are elevated in Tg2576 mice with HHcy. A) Representative western blots of Herp, Bip, and Hsp90 in brain homogenates from Ctrl group ($n = 7$) or Met group ($n = 6$). B) Densitometric analyses of the immunoreactivities to the antibodies shown in panel A. C) Herp mRNA levels relative to β -actin in brain homogenates from Ctrl group or Met group (open bars: Ctrl group; closed bars: Met group). Values represent mean $\pm$ S.E.M, * $P < 0.05$.

Fig. 2.

Herp protein and mRNA levels in a genetically-induced HHcy mouse model. A) Representative western blots of Herp, Bip, and Hsp90 in brain homogenates from Control mice or Tg-278 *Cbs*^{-/-} mice. B) Densitometric analyses of the immunoreactivities to the antibodies shown in panel A. C) Herp mRNA levels relative to β-actin in brain homogenates from control mice ($n = 4$) or Tg-278 *Cbs*^{-/-} mice ($n = 3$) (open bars: Ctrl group; closed bars: Tg-278 *Cbs*^{-/-} group). Values represent mean $\pm$ S.E.M.

Fig. 3. Herp protein levels in CHO-A β PPsw cell after homocysteine incubation. A) Representative western blots of Herp in CHO-A β PPsw cell lysate from control group or homocysteine incubated group. B) Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (open bars: Ctrl group; closed bars: homocysteine incubated group). Data were pooled from 3 experiments which had at least 3 samples in each group. Values represent mean $\pm$ S.E.M.

Fig. 4. Herp over-expression in CHO-AβPPsw cell did not affect AβPP protein or Aβ levels. A) Representative western blots of AβPP and Herp in CHO-AβPPsw cell lysate after transfection with either Herp cDNA or vehicle cDNA. B) Aβ₄₀ and Aβ₄₂ levels in the cell medium from these two groups (open bars: vehicle transfected group; closed bars: Herp transfected group). Data were pooled from 3 experiments which had at least 3 samples in each group. Values represent mean ± S.E.M.

Fig. 5. Herp over-expression in HEK293-AβPP cell did not affect AβPP protein or Aβ levels. A) Representative western blots of AβPP and Herp in HEK293-AβPP cell lysate after transfection with either Herp cDNA or vehicle cDNA. B) Aβ₄₀ and Aβ₄₂ levels in the cell medium from these two groups (open bars: vehicle transfected group; closed bars: Herp transfected group). Data were pooled from 3 experiments which had at least 3 samples in each group. Values represent mean ± S.E.M.