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Diet-Induced Hyperhomocysteinemia Increases Amyloid-β Formation and Deposition in a Mouse Model of Alzheimer's Disease

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Abstract

Hyperhomocysteinemia (HHcy) has been recognized as a risk factor for developing Alzheimer's disease (AD). However, its underlying molecular mechanisms are still elusive. Here we show that HHcy induces an elevation of amyloid beta (Aβ) levels and deposition, as well as behavioral impairments, in a mouse model of AD-like amyloidosis, the Tg2576 mice. This elevation is not associated with significant change of the steady state levels of the Aβ precursor protein (APP), β - or α-secretase pathways, nor with the Aβ catabolic pathways. By contrast, HHcy significantly reduces glycogen synthase kinase 3 (GSK3) Ser21/9 phosphorylation, but not total GSK3 protein levels. Similar results are obtained in brains homogenates from a genetic mouse model of HHcy. *In vitro* studies show that homocysteine increases Aβ formation, reduces phosphorylated GSK3 levels, without changes in total APP and its metabolism, and these effects are prevented by selective GSK3 inhibition. Overall, these data support a potential link between GSK3 and the pro-amyloidotic effect of HHcy *in vivo* and *in vitro*.

Keywords

Amyloid metabolism; amyloid β; Tg2576 mice; glycogen synthase kinase 3

INTRODUCTION

Alzheimer's disease (AD) is the most common form of neurodegenerative disease with dementia in the elderly, affecting approximately 6-8% all persons aged >65 years [1]. While only a minority of AD cases are caused by missense mutations in genes for either the Aβ precursor protein (APP) or Presenilin-1 and -2, the cause of sporadic AD remains unclear, and a combination of environmental and genetic factors with epigenetic events has been implicated [2]. Epidemiologic and clinical studies have revealed that elevated blood homocysteine levels, also called hyperhomocysteinemia (HHcy), is a risk factor for sporadic

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

AD [3]. Homocysteine is a non-protein sulfur-containing amino acid and intermediate product in the methionine cycle, whose normal levels in the body are maintained by its re-methylation to methionine in a reaction that requires the availability of dietary folate, vitamin B6 and B12 [4]. A diet with excessive methionine, or genetic alterations in certain enzymes of the methionine cycle increase homocysteine levels *in vivo* [5, 6].

The understanding of the molecular relationship between HHcy and AD pathogenetic mechanism(s) may provide important clues for the treatment or prevention of AD. Several potential mechanisms underlying the deleterious effect of HHcy in the brain have been proposed. These include oxidative stress [7], alterations in DNA methylation [8], DNA damage [9], and activation of NMDA receptors [10]. Another potential biological link between HHcy and AD, which has not been fully investigated, is an alteration of the APP metabolic pathway(s).

Previously, it was shown that crossing heterozygous cystathionine-β-synthase (Cbs) mutant mice, which spontaneously develop HHcy, with a transgenic mouse model of AD-like amyloidosis resulted in higher levels of brain Aβ peptides [11]. In that study, the authors reported no change in the β-secretase (BACE) levels. Thus, the mechanism by which HHcy modulates Aβ formation and deposition *in vivo* remains to be established.

In the present study, we used a different AD-like amyloidosis mouse model, the Tg2576 mice [12], in which HHcy was induced by feeding them with a diet containing high levels of methionine (Hofmann, *et al.* 2001). Compared with control mice, we observed that mice with HHcy had significantly higher Aβ levels and deposition and significant behavioral impairments. These Aβ changes were not associated with alterations of total APP, or its metabolic pathways, i.e. the β- and α-secretase, or Aβ catabolic pathways. While HHcy did not modify total levels of GSK3α/β , it resulted in a significant decrease in the GSK3 Ser21/9 phosphorylation levels, which are known to influence Aβ formation [13]. These results were confirmed in brain homogenates from a genetic model of HHcy, i.e. the Tg-278*Cbs*$^{-/-}$ mice [5]. Finally, *in vitro* studies showed that homocysteine increases Aβ formation whereby inducing a significant reduction of phosphorylated GSK3 levels, but without affecting total APP and GSK3 protein levels, and that selective inhibition of GSK3 reverses these effects. Taken together, our findings demonstrate a pro-amyloidotic effect of HHcy and suggest a possible involvement of GSK3 in this process.

MATERIALS AND METHODS

Tg2576 Mice and Diet Treatments

All animal procedures were approved by the Institutional Animal Care and Usage Committee. Tg2576 transgenic female mice expressing hAPP with the Swedish mutation (K670N/M671L) [12] were genotyped by polymerase chain reaction analysis using tail DNA and were kept in a pathogen-free environment, on a 12-hour light/dark cycle with ad libitum access to food and water.

Starting at 8 months of age, mice were randomized to two diets: standard rodent chow enriched in methionine (7.7g/Kg) or vehicle. Diets were custom-made, prepared by a commercial vendor (Harlan Teklad, Madison, WI), and matched for kilocalories [6]. Mice were sacrificed after 7 months on the diets at an age of 15 months.

After sacrifice, animals were perfused with ice-cold 0.9% PBS containing 10 mM EDTA, brains removed and dissected in two halves by midsagittal dissection. The left hemibrain was used for biochemistry assays; the right hemibrain was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.6) over night for immunohistochemistry studies. Separate groups of

Tg2576 mice following the same methionine-enriched diet (n=7) and regular chow (n=9) protocol were used for behavioral testing.

Tg-278Cbs$^{-/-}$ Mice

Tg-278*Cbs*$^{-/-}$ mice were generated on a C57B6 background as described before [5]. Three 12-month-old Tg-278*Cbs*$^{-/-}$ and age-matched C57B6 mice were sacrificed and their brains harvested as described above for biochemistry analyses.

Immunohistochemistry

Immunostaining procedures were performed as previously reported by our group [14, 15]. Coronal brain sections from levels between habenular nucleus and the posterior commissure were subjected to quantitative analysis. Every eighth section (8-10 sections per animals) was examined in a coded fashion and altogether in a single experiment. Light microscopic images from the hippocampus and somatosensory cortex were captured and used to calculate the area occupied by Aβ-immunoreactivity using the software Image-Pro Plus for Windows version 5.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). The threshold optical density that discriminated staining from background was determined, and kept constant for all quantifications. The area occupied by Aβ-immunoreactivity was measured by the software and divided by the total area of interest to calculate the percentage area occupied by Aβ-immunoreactivity. Analyses were always performed in a coded fashion.

Biochemical Analyses

Sequential extractions of brain homogenates in RIPA and then formic acid (FA) were performed as previously described [16]. For measuring Aβ1-40 and Aβ1-42 levels, sensitive sandwich ELISA kits were used (IBL America, Minneapolis, MN, USA). Analyses were always performed in duplicate and in a coded fashion. Homocysteine levels were assayed by using the "Abbott Homocysteine assay" with a fluorescence polarization immunoassay on the IMx® analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Western Blot Analyses

RIPA fractions of brain homogenates were used for Western blot analyses. Samples were electrophoresed on Tris-glycine polyacrylamide gels and pre-casted gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to nitrocellulose membranes. Antibodies and dilutions used for western blots are presented in supporting information.

CHO-APPsw Cell and *In Vitro* Study

CHO-APPsw cells stably transfected with human APP Swedish mutation were previously described [17]. Cells were maintained in McCoy's Medium (supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomycin) containing 200μg/ml G418, and treated with 500μM DL-homocysteine (Fluka Chemical, Milwaukee, WI, USA) for 4 days. On the third day, cell media were changed and fresh DL-homocysteine added. When needed, 30nM of the GSK3 inhibitor, 6-bromoindirubin-3′-oxime (BIO) (EMD Chemical Inc., Madison, WI, USA), or its inactive analog, 1-Methyl-BIO (MeBIO) (EMD Chemical Inc., Madison, WI, USA), were added to the cells at the same time with the homocysteine. Aβ levels in the medium were measured by a commercially available ELISA kit (IBL America, Minneapolis, MN, USA). Cell lysates were extracted with RIPA buffer and used for western blot analyses.

Behavioral Tests

Pre-pulse inhibition (PPI) and acoustic startle response were investigated in Tg2576 mice fed with diet (n=7) or regular chow (n=9) following a previously published protocol [18]. The details of the tests are provided in supplemental materials.

Data Analysis

Data analyses were performed using SigmaStat and SPSS. A 3 (PPI intensity) × 2 (diet) repeated measures ANOVA was used to examine the effect of the HHcy on PPI. An 8 (startle stimulus intensity) × 2 (diet) repeated measures ANOVA was used to examine the effect of HHcy on the acoustic startle response. Statistical comparisons between the different treatment groups in other analyses were performed by one way ANOVA. Values in all figures represent mean ± S.E.

RESULTS

HHcy and Aβ Formation

To investigate the effect of high circulating levels of homocysteine on Aβ formation, we induced HHcy in the Tg2576 mice by a well established dietary intervention model [6]. Starting at 8 months of age, animals were fed standard rodent chow enriched in methionine, or vehicle for seven months. During the study all mice gained weight as expected, and at the end of the treatment body weight, total plasma cholesterol and triglycerides levels were not significantly different between the two groups (Table 1). Confirming previous studies and the compliance with the diet, we found that, compared with control group (Ctrl), mice receiving the enriched diet (Diet) had a significant increase in plasma homocysteine levels (Diet: 35.4 ± 7.39 μM versus Ctrl: 6.33± 1.41 μM, $p<0.01$).

When compared with controls mice with HHcy had higher levels of Aβ peptides. In particular, we observed a significant increase in the formic acid (FA) fraction for both Aβ1-40 and Aβ1-42 in the hippocampus, and in the RIPA fraction for Aβ1-42 in the cortex (Fig. 1). Serial brain sections from the two groups of mice were immunostained with 4G8, an anti-Aβ antibody reactive to amino acid residues 17-24, and the positive immunoreactive areas were calculated. As shown in (Fig. 2), we found that, compared with the control group, the Diet group had a significant increase in Aβ immuno-reactivity in the hippocampus (Diet: 1.51% ± 0.26%, Ctrl: 0.64% ± 0.12%; $p<0.01$); and the somatosensory cortex (Diet: 1.98% ± 0.43%, Ctrl: 0.90% ± 0.14%; $p<0.05$).

HHcy and APP Metabolism

Having observed that HHcy increases Aβ formation and deposition, we next focused on possible mechanism(s) responsible for this effect.

First, we assessed the steady-state levels of APP and its cleavage products. Compared with brain homogenates from the control group, total APP levels were unaltered in the Diet group (Fig. 3). Similarly, β-secretase pathway represented by the β-site APP cleaving enzyme (BACE-1), and secreted APPβ (sAPPβ) did not differ between the two groups (Fig. 4). Mice with HHcy did not show any significant alteration of the α-secretase pathway either, as measured by the secreted APPα (sAPPα), ADAM10 and C-terminal fragment-α (CTF-α; C83) (Fig. 3). Further, no difference was found for PS1 and Nicastrin protein levels, two major components of the γ-secretase complex (Fig. 3). By contrast, C-terminal fragment-β (CTF-β; C99) level was statistically significant lower in the brains of the Diet group, compared to controls (Fig. 3).

Next, we analyzed two of the major proteases involved in the catabolism of Aβ , i.e., insulin-degrading enzyme (IDE) and neprilysin [19]. Steady-state protein levels of IDE and neprilysin measured by western blot were similar between the two groups of animals (Fig. S1). The same was also valid for apoE and glial fibrillary acidic protein (GFAP) levels (Fig. S1).

HHcy and Brain GSK3

To further elucidate the mechanism underlying the increase in Aβ levels and deposition observed in mice with HHcy, we used semi-quantitative western blot analysis to measure GSK3 levels, a kinase enzyme that has been shown to alter Aβ formation whereby modulating the γ-secretase pathway [20]. As shown in (Fig. 5), we found that the total protein levels for GSK3α and GSK3β were not significantly different between the two groups. By contrast, compared with controls, brain homogenates from the Diet group had statistically significant lower GSK3α/β Ser21/9 phosphorylated levels (Fig. 5).This reduction was also associated with a significant decrease in Akt, a kinase known to regulate GSK3 phosphorylation state [21], and a trend toward decrease of its phosphorylated form at Thr308, which did not reach statistical significance. No significant difference was found between the two groups for 3-phosphoinositide-dependent protein kinase 1 (PDK1) levels, or its phosphorylated form at Ser241 (Fig. 6).

Behavioral Tests

Tg2576 mice were subjected to the PPI and acoustic startle response test, which are measures of sensorimotor gating [22], after 6 and 7 months on the diet. Although the results from the first run did not reveal a significant difference for both tests, we observed a trend towards difference between the two groups on the startle response amplitude (Fig. S2). However, at the end of their treatment (after 7 month on the diet), we observed a significant main effect of decibel level on PPI [$F(2,14) = 24.27$, $p < 0.01$] and a trend of main effect of diet on PPI [$F(1,14) = 2.92$, $p = 0.109$]. Further, we found that the HHcy group had a significant lower %PPI at 81dB level than the controls [$F(1,14) = 7.025$, $p < 0.05$] (Fig. 7A). Finally, we observed a significant main effect of decibel level on acoustic startle [$F(7,14) = 12.21$, $p < 0.01$], a significant interaction between dB level and diet [$F(7,14) = 3.07$, $p < 0.01$], and a trend of main effect of diet on acoustic startle[$F(1,14) = 3.489$, $p = 0.083$] between the two groups (Fig. 7B).

Genetically Induced HHcy and Brain GSK3

To further confirm the effect of HHcy on GSK3 phosphorylation state, brain homogenates from 12 months old mice genetically deficient in cystathionine-beta-synthase (Tg-278*Cbs*$^{-/-}$ mice), which as a result manifest HHcy [5], were analyzed. As shown in (Fig. 8), while we observed that total levels of GSK3α/β were unchanged, we found a significant reduction in phosphorylated GSK3α/β levels (phosphorylated at Ser21/9).

In Vitro Effect of Hcy on Aβ Levels and GSK3

The results accumulated so far suggest that HHcy by modulating the phosphorylation state of GSK3 increases Aβ formation. To gain further support to this hypothesis, next we exposed CHO cells over-expressing APP Swedish mutant (CHO-APPsw) to homocysteine alone or in the presence of a specific GSK3 inhibitor. At the end of the treatment, conditioned media were collected for Aβ measurement and cell lysates assayed for APP metabolism. Under this condition, we found that compared with media obtained from vehicle-treated cells, Aβ peptides were increased in the supernatants of cells treated with 500μM homocysteine. In particular, while Aβ1-40 increase did not reach statistical significance, by contrast we observed that Aβ1-42 levels were significantly increased ($p <$

0.05) (Fig. 9). Under this condition, CTFs levels in the homocysteine-treated cells were significantly lower than in the vehicle-treated ones (Fig. 9). However, no difference in the steady state levels of total APP, sAPPβ or total GSK-3α/β was observed between cells treated with homocysteine or vehicle (Fig. 9). By contrast, we observed that phosphorylated GSK3α/β levels were significantly reduced in the presence of homocysteine (Fig. 9). Finally, addition of a specific GSK3 inhibitor (BIO) to homocysteine-treated cell prevented the increase of the Aβ formation without affecting Aβ basal levels (Fig. 10A). By contrast, its inactive analog (MeBIO) showed no effect on the Aβ increase when incubated with homocysteine-treated cells (Fig. 10A). Western blot analysis showed that the GSK3 inhibitor BIO, but not its inactive analog, reversed the homocysteine-induced reduction of CTFs and phosphorylated GSK3 without affecting total APP, sAPPβ and GSK3 protein levels (Fig. 10 B-E).

DISCUSSION

The present studies were designed to investigate the effect of a diet-induced HHcy on the amyloidotic phenotype of a transgenic mouse model of AD. An emerging body of literature supports the concept that there is an association between elevated levels of homocysteine and AD [23-26], and longitudinal studies show that HHcy doubles the risk for developing dementia and AD independently of several major confounders [27]. Although several potential mechanisms underlying the deleterious effect of HHcy in the central nervous system have been proposed, the interaction between HHcy and APP metabolism has not been fully elucidated [11].

To investigate this relationship, we used a dietary approach by feeding the Tg2576 mice with a diet containing high levels of methionine, which is known to results in HHcy [6]. Thus, at the end of the treatment period we found that, compared with the control group, mice receiving the enriched diet had elevated plasma homocysteine levels, which were within the range reported for elderly people (5.4 to 61.6 μM) in the Framingham study [3]. The diet-dependent HHcy associated with a significant increase in brain A peptide levels and deposition and behavioral deficits as assessed by PPI and startle responses. In search for possible mechanism(s) responsible for this *in vivo* effect, we investigated APP metabolic as well as Aβ catabolic pathways. First, we demonstrated that steady state levels of total APP were not different between the two groups, and that this was also the case for its two main metabolic pathways, i.e. the β- and α-secretase. Next, we focus on the γ-secretase pathway. To this end, PS1 and nicastrin steady-state levels, two major components of γ-secretase, were not altered by HHcy. To rule out other mechanisms responsible for the increased Aβ levels, we next assessed IDE, neprilysin and apoE levels, all of which have been involved in Aβ clearance [28]. No significant change in these proteins was observed between the two groups. However, since we did not assay for the activity of these enzymes (i.e. γ-secretase, IDE and neprilysin), we can not rule out the possibility that they were affected by HHcy.

GSK3 is a major serine-threonine kinase, which has been implicated in AD pathogenesis [20]. Since among other targets, this kinase has been shown to promote Aβ production [13], we tested the hypothesis that it was involved in the HHcy-induced Aβ increase in our model. Western blot analyses showed no difference in the total levels of GSK3α/β in brain homogenates from the two groups of mice. By contrast, we observed a statistically significant reduction in the phosphorylation state of GSK3α/β at Ser21/9 in brains from the HHcy group. This was also the case for Akt, a regulatory kinase which is upstream to GSK3 [21].

Tg2576 mice with HHcy showed significant deficit in PPI and a trend of lower acoustic startle response amplitude when compared with controls. We are confident that this result is

not secondary to a deficit in hearing of the mice tested since if there was one they would show more deficits when responding to the sound of lower dB rather than higher ones. Interestingly, the deficit in PPI, which indicates dysfunction of normal sensorimotor gating and/or attention [29], has been reported in AD patients [30] and AD mouse models [31, 32]. Since previous studies found that damage in the hippocampus leads to impairment in PPI performance [33], it is possible that the deficits we observed in the HHcy group is related to the higher Aβ levels detected in their hippocampi.

It is known that when GSK3α/β is phosphorylated on residue Ser21/9 by PKB/Akt, its enzymatic activity is reduced [34, 35]. Thus, the lower GSK3 phosphorylation levels we observed suggest that the activities of the kinase is elevated , which could lead to the Aβ elevation [13]. Interestingly, the relationship between HHcy and this kinase was also detected in brain homogenates from a genetic model of HHcy, where while there was no change in total levels of GSK3, we observed a significant reduction in GSK3α/β Ser21/9 phosphorylation levels.

To further support the role of GSK3 in the pro-amyloidotic effect of HHcy, we performed *in vitro* experiments in which we directly exposed cells to homocysteine. Under this condition, while no effect on total levels of APP was observed, cells produced significantly higher levels of Aβ peptides. Similar to the *in vivo* study, homocysteine treatment was associated with a significant decrease in CTFs, and a reduction in GSK3α/β (Ser21/9) phosphorylation levels, while total GSK3α/β remained unaltered. Importantly, all of these HHcy-induced effects were reversed by a selective GSK3 inhibitor, but not by its inactive analog.

Elevated homocysteine in the body leads to the elevation of many related metabolites and some of these metabolites, e.g. homocysteine thiolactone, can also inhibit the insulin-receptor signaling pathway which include Akt and GSK3 [36, 37]. Interestingly, a previous study found that homocysteine thiolactone reduces the phosphorylation of GSK3α/β at Ser21/9 induced by insulin [38], suggesting, as in our study, a molecular link between HHcy and GSK3 signaling pathway.

In summary, our study demonstrates that diet-induced HHcy results in a significantly increase in Aβ formation and deposition, and behavioral deficit in a transgenic mouse model of AD. The effect on Aβ formation associates with a reduction in GSK3 phosphorylation state supporting the hypothesis that this signaling pathway may be involved in the biological effect. Pharmacological modulation aimed at reducing the activity of this kinase could represent a novel therapeutic approach for subjects with HHcy, a known risk factor to develop AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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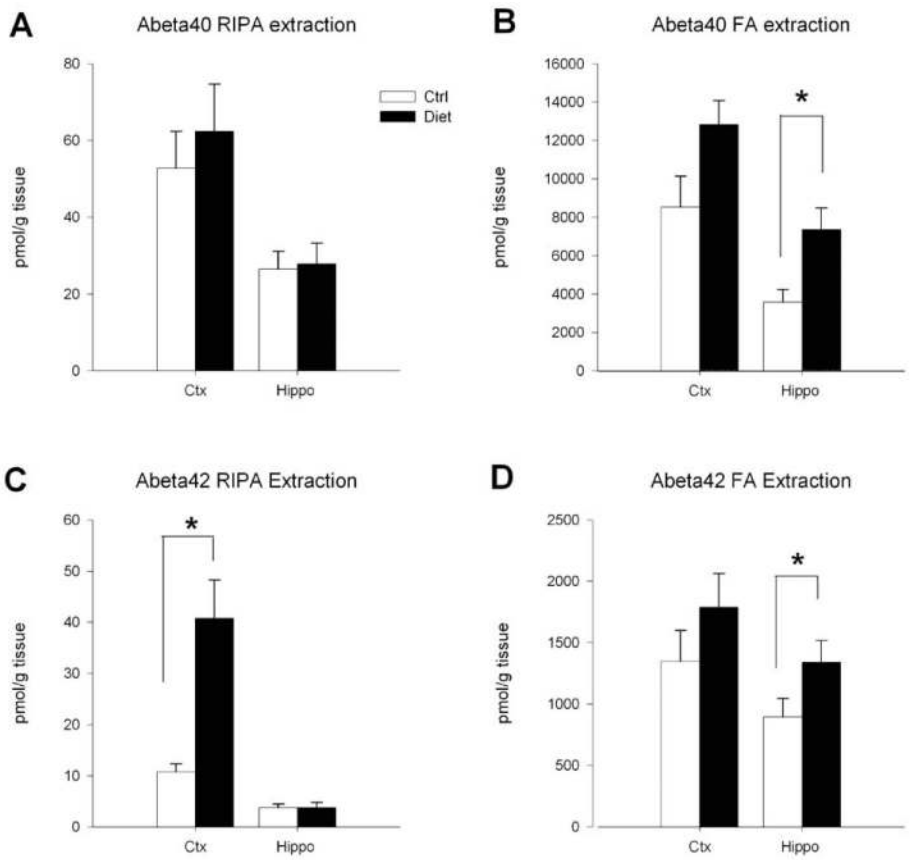

Fig. (1).

Diet-induced HHcy in Tg2576 mice results in increased Aβ peptide levels. RIPA-soluble (RIPA) and formic acid extractable (FA) Aβ1-40 **(A, B),** and Aβ1-42 **(C, D)** levels in the cortex (Ctx) and hippocampus (Hippo) from Tg2576 on a methionine- enriched diet (Diet) or vehicle (Ctrl) were measured by sandwich ELISA. Values represent mean ± S.E.M. (n = 6 per group). (*$P < 0.05$).

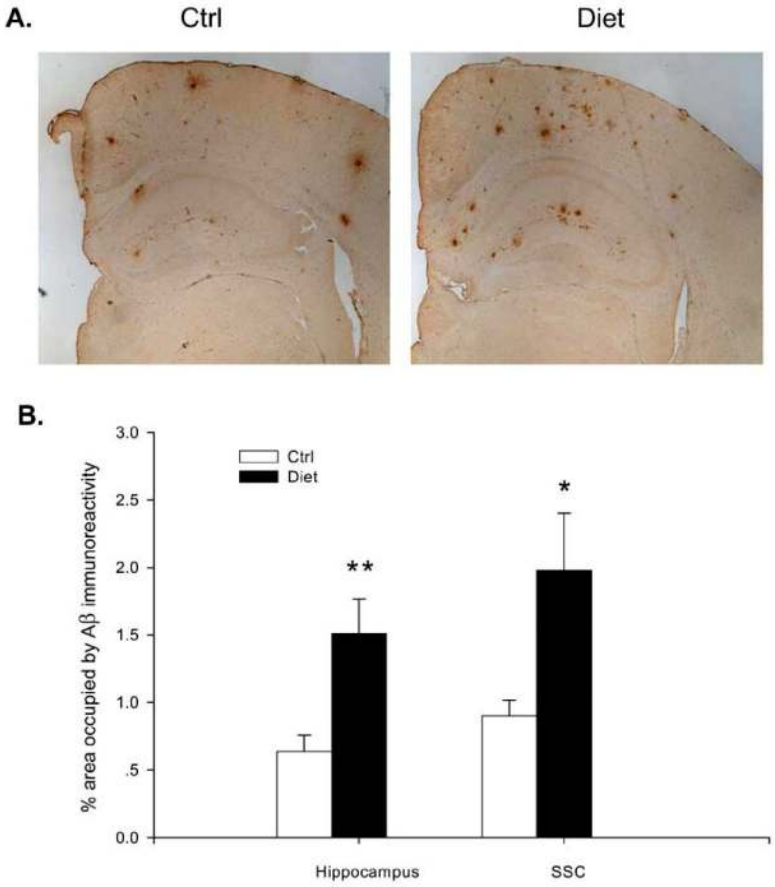

Fig. (2).

Diet-induced HHcy in Tg2576 mice results in increased Aβ deposition. **A)** Representative sections of brains of Tg2576 receiving enriched diet (Diet), or vehicle (Ctrl) immunostained with 4G8 antibody. **B)** Quantification of the area occupied by Aβ immunoreactivity in hippocampus and somatosensory cortex (SSC) of Tg2576 on methionine-rich diet (Diet group, n= 5), or control diet (Ctrl group, n= 6). Values represent mean ± S.E.M. (*$P < 0.05$, **$P < 0.01$).

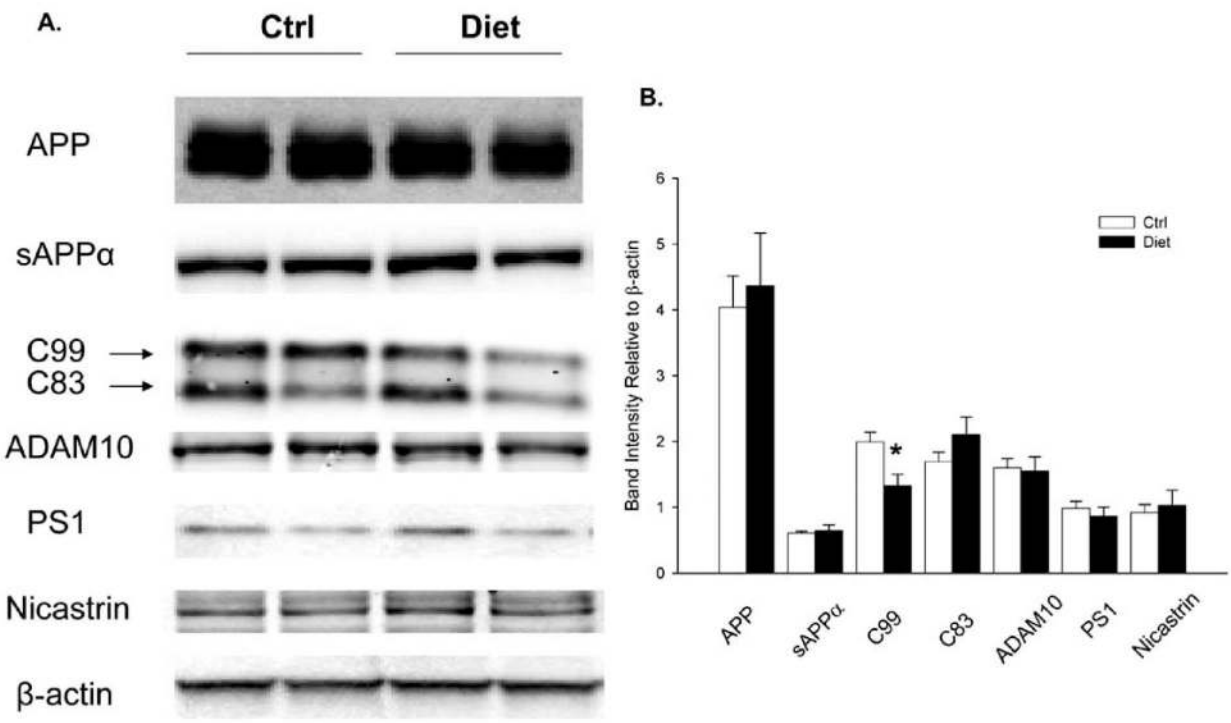

Fig. (3).

APP metabolism in Tg2576 mice with HHcy. **A)** Representative western blots of APP, sAPPα, CTFs (C99 and C83), ADAM10, PS1 and Nicastrin in brain homogenates from Diet group or Ctrl group. **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: Ctrl group; closed bars: Diet group). Values represent mean ± S.E.M. (**P* < 0.05).

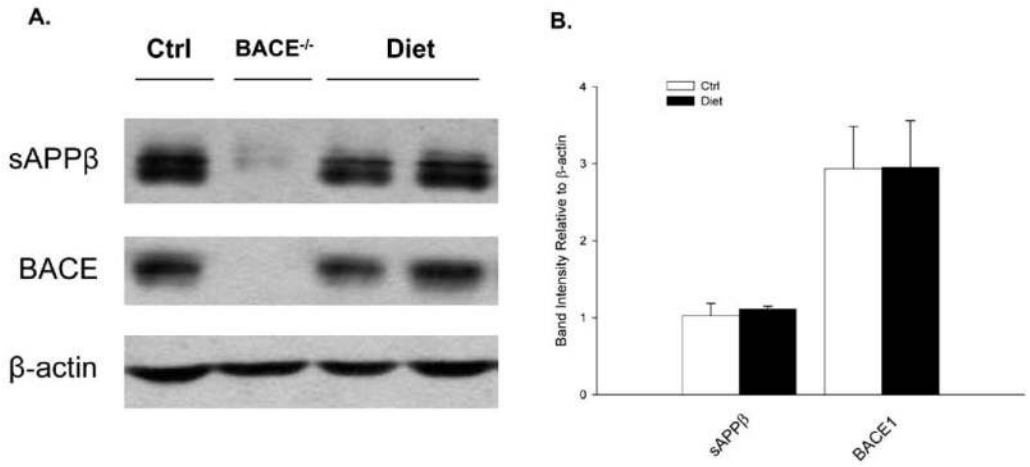

Fig. (4).

β-secretase pathway is unaltered in Tg2576 mice with diet-induced HHcy. **A)** Representative western blots of sAPPβ, BACE in brain homogenates from Tg2576 on control diet (Ctrl) or methionine-enriched diet (Met). **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: Ctrl group; closed bars: Diet group). Values represent mean ± S.E.M.

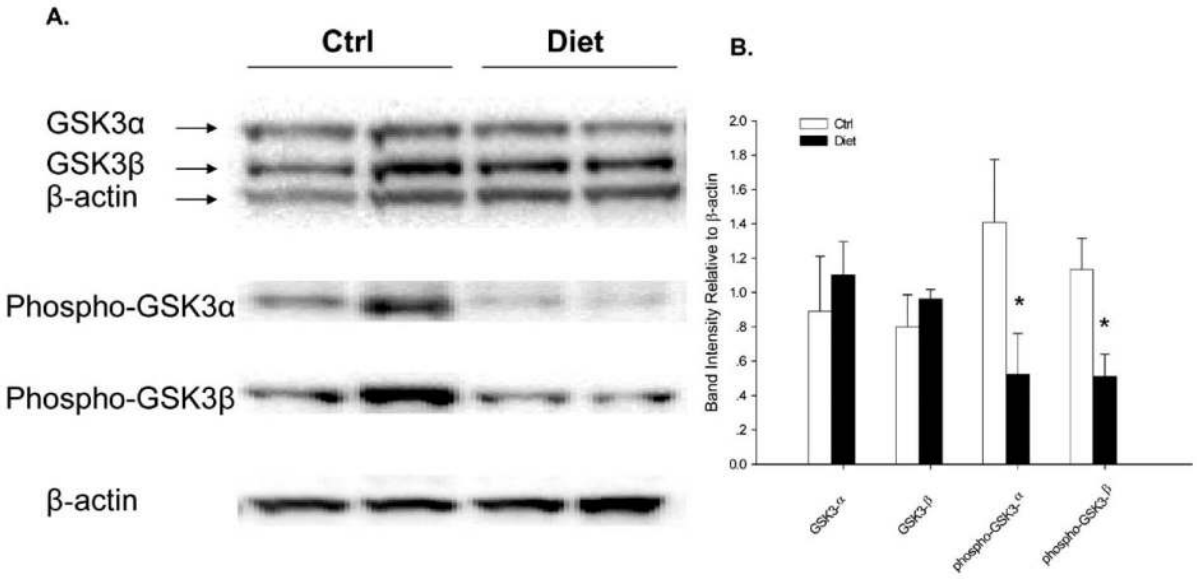

Fig. (5).

Effects of HHcy on GSK3 levels in Tg2576 brain homogenates. **A)** Representative western blots of total GSK3α/β , and phosphorylated GSK3α/β on Ser21/9, in brain homogenates from Tg2576 mice. **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: Ctrl group; closed bars: Diet group). Values represent mean ± S.E.M. (**P* < 0.05).

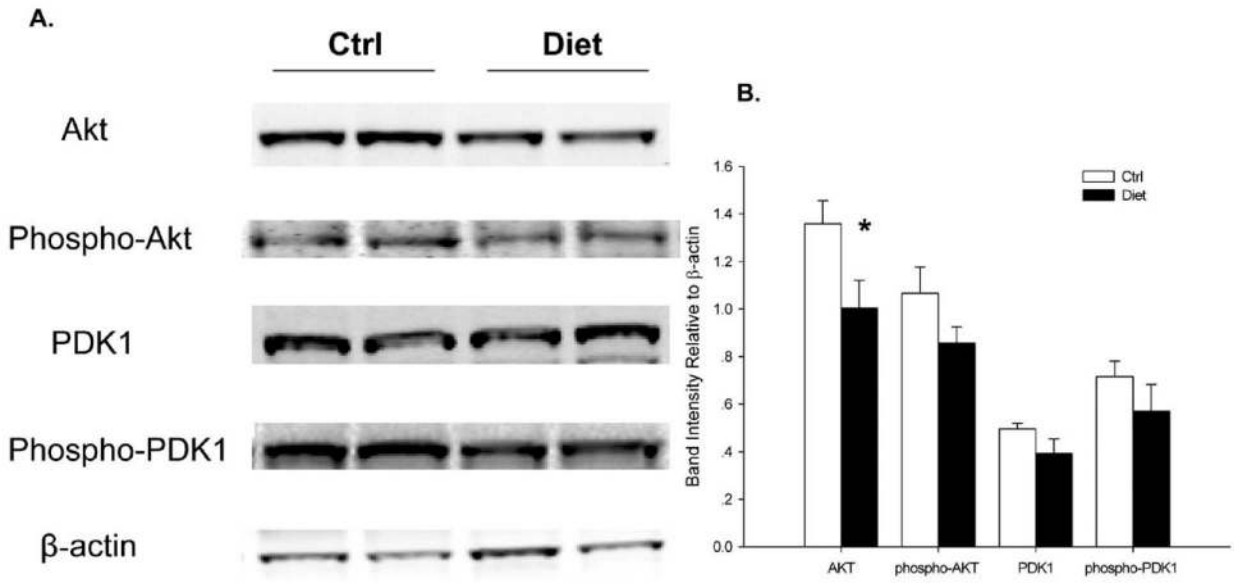

Fig. (6).

Effects of HHcy on Akt and PDK1 levels in Tg2576 brain homogenates. **A)** Representative western blots of total Akt, phosphorylated Akt (Thr308), PDK1 and phosphorylated PDK1 (Ser241) in brain homogenates. **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: Ctrl group; closed bars: Diet group). Values represent mean ± S.E.M. (*$P < 0.05$).

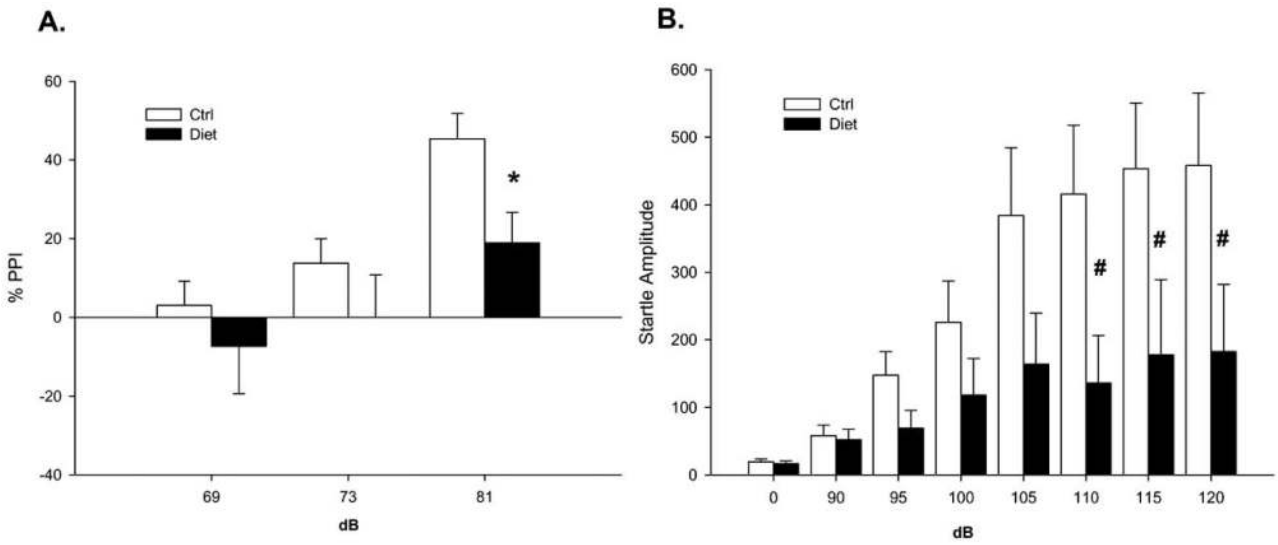

Fig. (7).

Effect of diet-induced HHcy on the pre-pulse inhibition (PPI) and startle responses in Tg2576 mice. **A)** Mice with HHcy (Diet) showed significantly lower performance on the PPI than controls (Ctrl) with pre-pulse intensities of 81dB. **B)**. They also showed a trend towards a lower startle amplitude with pulse intensities of 110, 115 and 120 dB when compared with controls. Values represent mean ± S.E.M. (* $P < 0.05$; # $0.05 < P < 0.10$).

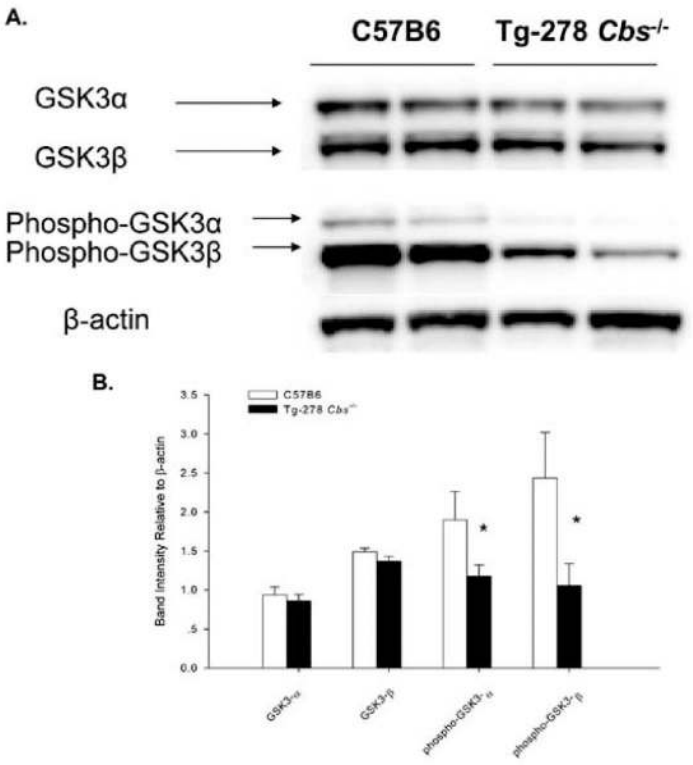

Fig. (8).

Effect of genetic-induced HHcy on GSK3. **A)** Representative western blots of brain homogenates from C57B6 mice and Tg-278*Cbs*$^{-/-}$ mice probed with specific antibodies against total GSK3α/β, and phosphorylated GSK3α/β on Ser21/9, respectively. **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: C57B6 control mice; closed bars: Tg278*Cbs*$^{-/-}$ mice). Values represent mean ± S.E.M. (*P < 0.05).

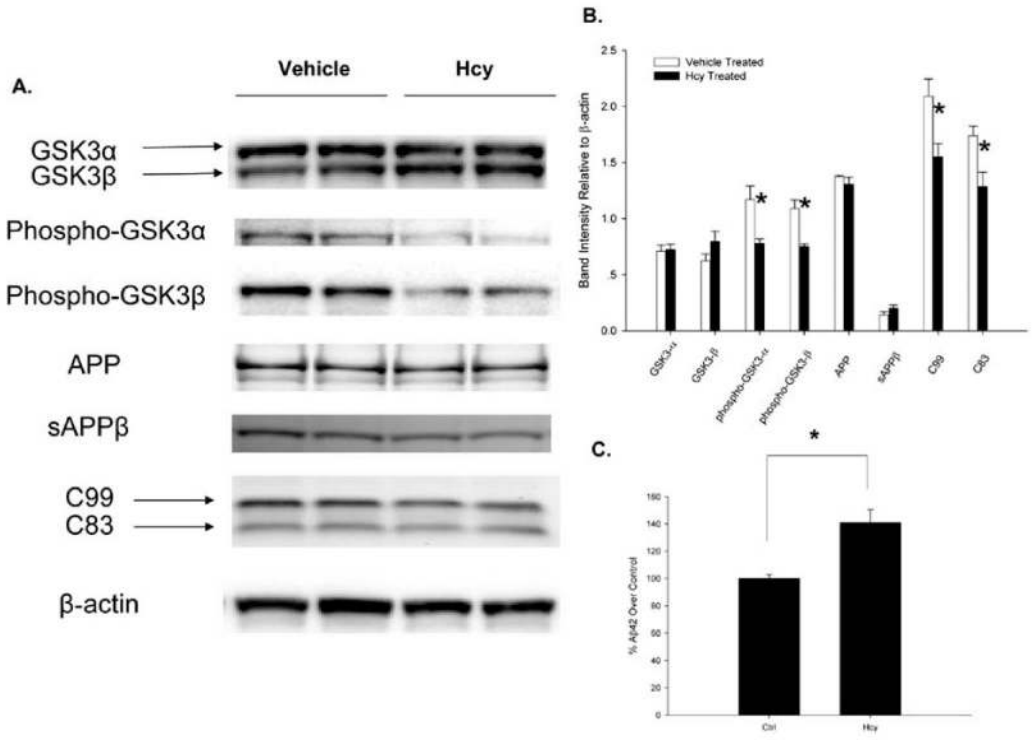

Fig. (9).

Effects of homocysteine on APP metabolism in homocysteine-treated CHO-APPsw cells. **A)** Representative western blots of cell lysates probed with specific antibodies against total GSK3α/β, phosphorylated GSK3α/β on Ser21/9, APP, sAPPβ, CTFs (C99 and C83). **B)** Densitometric analyses of the immunoreactivities shown in panel A (open bars: vehicle-treated cells; closed bars: homocysteine (Hcy)-treated cells). **C)** Aβ levels in the supernatants from Hcy-treated CHO-APPsw cells. (* $P < 0.05$)

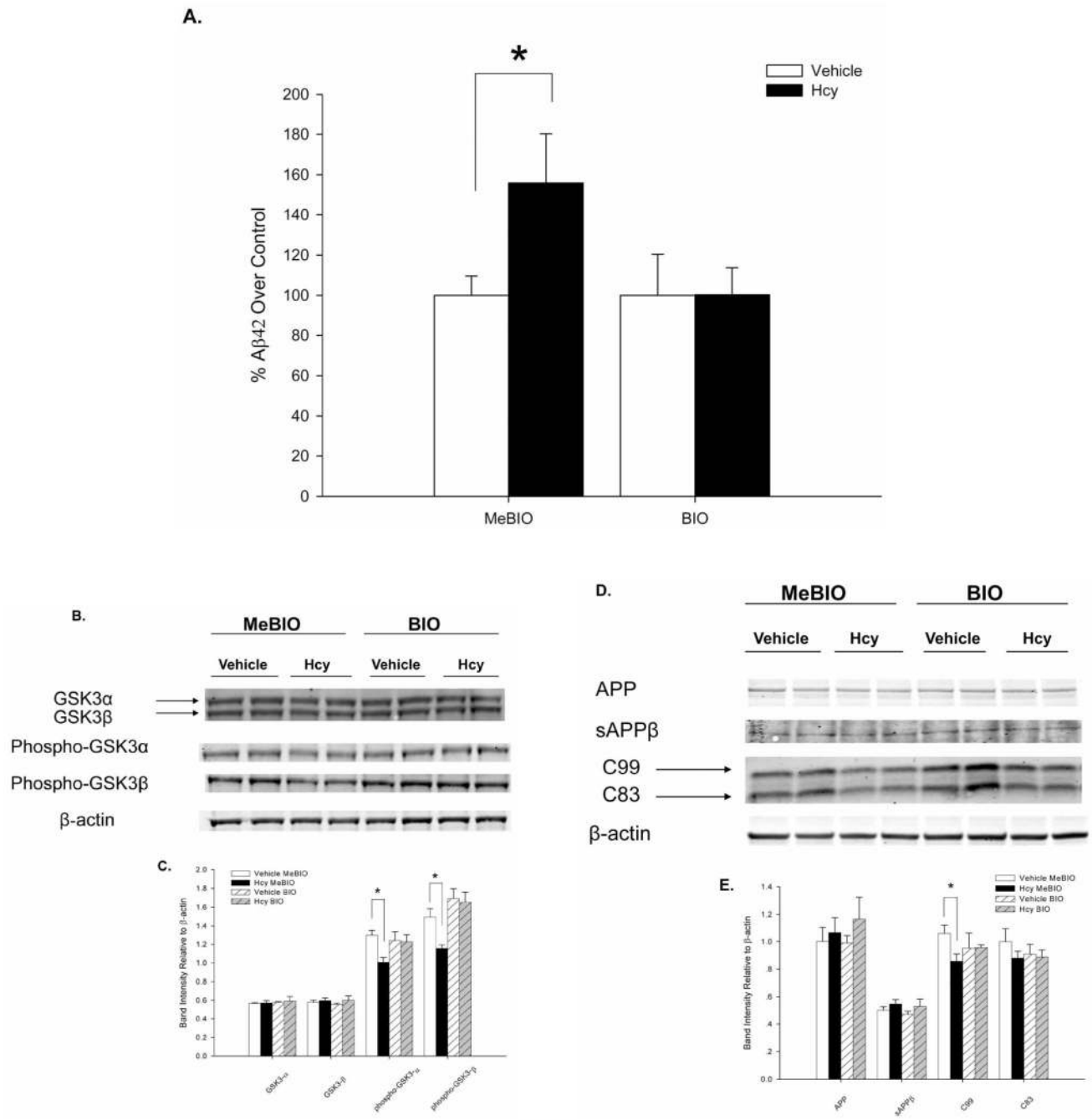

Fig. (10).

Effect of GSK3 inhibition on APP metabolism in homocysteine-treated CHO APPsw cells. **A)** Hcy-induced Aβ increase was blocked in the presence of GSK3 inhibitor BIO, but not by its inactive analog MeBIO. **B)** Representative western blots from the same cells probed with specific antibodies against total GSK3α/β, and phosphorylated GSK3α/β on Ser21/9. **C)** Densitometric analyses of the immunoreactivities shown in panel B. **D)** Representative western blots from the same cells probed with specific antibodies against total APP, sAPP , CTFs (C99 and C83). **E)** Densitometric analyses of the immunoreactivities shown in panel

D. (open bars; vehicle+MeBIO; closed bars: Hcy+MeBIO; open crossed bars: vehicle+BIO; closed crossed bars: Hcy+BIO). Values represent mean ± S.E.M. (*P < 0.05)

Table 1

Effect of Seven Months Exposure of Tg2576 Mice to a Methionine-Rich Diet on Body Weight, Plasma Total Cholesterol and Triglycerides Levels. (Results are Mean ±S.E.)

	Control	Diet
Weight (g)	29 ± 2.2	30 ± 2.5
Total Cholesterol (mg/dL)	150 ± 24	145 ± 29
Triglycerides (mg/dL)	115 ± 20	119 ± 18