Betaine Induced Autophagy to Protect Neurons Against Amyloid Beta in Alzheimer's Disease

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Research Article

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Abstract

Background: Alzheimer disease is a neurodegenerative disease, which is accompanied by the accumulation of amyloid beta and tau hyper phosphorylation. The objective of this study is to investigate amyloid-beta clearance through autophagy in HMC3 cells by betaine.

Methods: In this study HMC3 cells were used. LC3, P62, p-AKT, p-MTOR, Beclin1, ATG 5, ATG 7, ATG 12 and beta-actin were studied by western blot. LC3 was studied by confocal microscopy. Neuroprotection and cell viability were assessed by MTT assay. Confocal microscopy and ELISA studied amyloid-beta clearance. Student's T-test was used to compare the treated vs untreated groups.

Results: We have identified betaine an autophagy inducer in microglia HMC3 cells. We have further tried to establish the mechanism of betaine for autophagy induction. Betaine was able to induce autophagy through the PI3K/AKT pathway. Further, we investigated the clearance of amyloid-beta by betaine through autophagy induction. Betaine was also able to reduce the toxicity of amyloid-beta in human SHSY-5Y cells. A known down of LC3 reverses the protective effect of betaine against amyloid-beta in differentiated SHSY5Y cells.

Conclusion: Betaine induced autophagy, clears amyloid-beta and protects SHSY-5Y cells against amyloid-beta. Based on these data, betaine can be further studied for anti-Alzheimer therapy.

Background

Alzheimer disease (AD) is one form of the dementia of an unknown etiology. There are two main culprits in Alzheimer's disease like tau hyper phosphorylation and accumulation of amyloid beta (A β) (1). There are two types of Alzheimer's disease like familial and sporadic Alzheimer's disease. In familial Alzheimer's disease there is one mutation in one of the genes, which forms the truncated proteins known as A β plaques. In sporadic Alzheimer's disease, there is an imbalance between the A β production and clearance that produced aggregation of toxic proteins known as A β (2). Once amyloid beta is accumulated into the cells it produced reactive oxygen species, which leads to the distraction of neurons (3). There are four drugs available in the market, which gives the symptomatic treatments to the patients, but there is no drug, which cures the disease (4). Antibodies based clinical trials targeting amyloid beta was failed but recently one of the antibody based clinical trial targeting amyloid beta have shown significant improvement in the memory of the patients (5, 6). Therefore, targeting clearance of amyloid beta may be a good clinical target for the treatment of Alzheimer's disease. There are various pathways by which amyloid beta is cleared from the brain, among them autophagy plays an important role in amyloid beta clearance (7). Autophagy is a cellular lysosomal degradation pathway by which it sequesters the damaged organelles and protein aggregates into a double membrane structure known as autophagosomes (8). These double membrane structures fuse with the lysosome, which contains acid hydrolases for degradation (9). The accumulation of A β by autophagy impairment worsens the disease pathology (10). In transgenic mouse model of Alzheimer's disease the expression of beclin1 was reduced

that hampers autophagy, which leads to the accumulation of amyloid beta and neurodegeneration. The genetic manipulation of beclin1 significantly improved the pathology in AD mouse model (11). Various reports suggested that the pharmacological induction of autophagy clears amyloid beta and protects the neurons against amyloid beta (12–17). It was found that natural products play an important role for autophagy induction in neurodegenerative diseases (18). In this study, betaine was able to induce autophagy in HMC3 cells. Betaine is a modified amino acid with glycine containing three methyl groups' acts as a methyl donor in various metabolic pathways. Betaine was reported to treat the rare genetic cause of homocystinuria (19). Here we are reporting for the first time that betaine-induced autophagy and clears amyloid beta in microglial HMC3 cells. We further investigated that betaine inhibits PI3K/AKT pathway for the induction of autophagy and protect differentiated SHSY-5Y cells against A β .

Results

Betaine increased the expression of LC3-II in HMC3 cells

To explore whether betaine induces autophagy in HMC3 cells, we treated betaine at a concentration of 1, 5 and 10 mM for 24 h. It was shown that betaine was able to induce autophagy at 1, 5 and 10 mM as detected by the conversion of LC3-I into LC3-II and decreased the expression of SQSTM1 through western blot (Figure 1A, B). Based on the autophagy induction, betaine 5 mM was chosen for further studies. These results were further validated through confocal microscopy in which the expression of LC3 puncta was significantly increased in betaine treated HMC3 cells compared to control (Figure 1C, D).

Betaine activates PI3K/AKT pathway to induce autophagy in HMC3 cells

We further investigated the mechanism of autophagy induction treated with betaine in HMC3 cells. Betaine was able to increase the expression of autophagic proteins like beclin1, ATG5, ATG7, ATG12 in HMC3 cells. Furthermore, we have tried to decipher the mechanism of autophagy induction by betaine. Interestingly, it was shown that the expression of p-AKT and mTOR was inhibited in a concentration dependent manner (Figure 2). These results suggested that betaine was able to induce autophagy through PI3K/AKT pathway.

Betaine clears amyloid beta in microglia HMC3 cells

In Alzheimer's disease amyloid beta accumulates into the cells, which leads to the death of neurons. With this view, we have co-treated HMC3 cells with hilyte amyloid beta 555 (10 mM) and betaine at a concentration of 1, 5 and 10 mM. Confocal microscopy analysis of amyloid beta in betaine treated cells revealed the reduced fluorescence of hilyte A β_{1-42} 555 as compared with hilyte A β_{1-42} 555 alone (Figure

3A). These results were further collaborated with ELISA in which the absorbance of amyloid beta was reduced in betaine treated cells compared with amyloid beta alone (Figure 3B).

Amyloid beta induced toxicity was reversed with betaine

The accumulation of Ab produced the reactive oxygen species and leads towards neurodegeneration. Betaine was able to clear amyloid beta in HMC3 cells, there it was possible that betaine can able to protect neurons against amyloid beta. We treated differentiated SHSY-5Y cells with amyloid beta in presence and absence of betaine. Surprisingly, it was shown that betaine was able to increase the viability of differentiated SHSY-5Y cells that was reduced with amyloid beta alone (Figure 4A). The differentiation of SHSY-5Y cells was checked by western blot (Figure 4B). Therefore, our data revealed that betaine was able to protect the differentiated SHSY-5Y cells against amyloid beta induced toxicity.

Knockdown of LC3 hampers the neuroprotective effect induced by betaine

In order to validate the neuroprotective affect of betaine, we transfected differentiated SHSY5Y cells with siRNA LC3 and treated with amyloid beta in presence and absence of betaine for 24 h. It was shown that neuroprotection induced by betaine was hampered in siLC3-transfected cells (Figure 5A). The transfection efficacy was more than 60%, which was detected by immunoblotting (Figure 5B).

Discussion

Defective clearance mechanism leads to the accumulation of A β in AD (20). Autophagy is an important mechanism by which A β is cleared from the brain (21). Hampered autophagy leads the accumulation of intracellular as well as extracellular amyloid beta into the brain (22). The pharmacological intervention of autophagy leads the clearance of amyloid beta, protects neurons and increased the cognitive abilities in AD mouse model (12–17). With this view, we have tried to find out the potential of autophagy by betaine to clear amyloid beta in microglia HMC3 cells. Microglial cells play a crucial role in amyloid beta clearance because these cells have higher phagocytosis capacity to uptake amyloid beta into the surroundings (23). These specialized cells have specific receptors like TREM and CD33 for uptake of amyloid beta (24). Autophagy is regulated by the activation of AMPK or inhibition of PI3K/AKT pathway (25). We have further tried to find the mechanism of autophagy induction by betaine. Interestingly, betaine was able to inhibit PI3K/AKT pathway by inhibiting p-AKT (ser473) in HMC3 cells. The other important proteins of autophagy like beclin1; ATG5, ATG7 and ATG12 were increased by betaine. We further investigated betaine for clearance of amyloid beta in HMC3 cells. Surprisingly, we have observed that betaine was significantly able to clear amyloid beta in HMC3 cells in a concentration dependent manner. Amyloid beta accumulation generates reactive oxygen species and induces neurotoxicity in Alzheimer's disease (26). Interestingly, it was shown that betaine was able to reduce toxicity induced by

amyloid beta in differentiated SHSY-5Y cells, which was confirmed by knockdown of LC3 in betaine treated cells. In conclusion, betaine was able to induce autophagy through inhibition of PI3K/AKT pathway, clears amyloid beta in human microglia HMC3 cells and provides neuroprotection against amyloid beta induced toxicity in differentiated SHSY-5Y cells.

Conclusions

Betaine induced autophagy, clears amyloid-beta and protects SHSY-5Y cells against amyloid-beta. Knockdown of LC3 abrogates the effected induced by amyloid beta in differentiated SHSY-5Y cells. Based on these data, betaine can be further studied as an anti-Alzheimer therapy.

Methods

Reagents:

Minimum Essential Medium (MEM), Roswell Park Memorial Institute (RPMI), Dulbecco's Minimal Essential medium (DMEM), retinoic acid, 4, 6-diamidino-2-phenylindole (DAPI) Penicillin G, TEMED, triton X-100, glycine, sodium bicarbonate, tween 20, Phosphate buffered saline (PBS), sodium fluoride, EDTA, A β ₁₋₄₂, sodium orthovanadate, paraformaldehyde, skimmed milk, Radioimmunoprecipitation assay buffer (RIPA), trypsin, sodium pyruvate, sodium dodecyl sulphate, Streptomycin sulphate, rapamycin, bovine serum albumin (BSA) dimethylsulfoxide (DMSO), ammonium persulfate, HEPES, glycerol, sodium chloride, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), , anti-LC3 and anti-SQSTM1 antibodies were purchased from Sigma-Aldrich. Lipofectamine, fetal bovine serum (FBS), Human A β ₁₋₄₂ kit, were obtained from Invitrogen. A β ₁₋₄₂-HiLyte FluorTM 555 were purchased from Anaspec. ECL-kit and PVDF membrane were purchased from Millipore. Bradford and protein marker ladder was obtained from Bio-rad. Antibodies were purchased from Cell Signalling Technology.

Cell culture:

Human microglial HMC3 cells were obtained from American Type Culture Collection (ATCC). HMC3 cells were grown in RPMI media and Human SHSY-5Y cells maintained in MEM media complemented with 100 mg/L streptomycin, 80 mg/L penicillin G and 10% FBS. These cells were grown in an incubator containing CO₂ (5 %) and 95 % humidity. SHSY-5Y cells were differentiated into 5% FBS containing 10 mM retinoic acid for 7 days and neuronal markers were checked through western blot.

Western blot:

Microglial HMC3 cells grown into 60 mm dishes and were treated with betaine for 24 h at a concentration of 1, 5 and 10 mM. The cells were washed with PBS followed by lysis with RIPA bufffer having sodium orthovanadate, cocktail and sodium fluoride for 1 h. These samples were collected after centrifugation

and proteins were estimated by bradford reagent followed by the standard curve by using BSA. Equal amount of proteins were loaded into each wells and were run for 2 hours in SDS page buffer at 100V. These samples were transferred into PVDF membrane at 100V for 2 h and the protein membranes were incubated into 5% skimmed milk for blocking. Primary antibodies were probed into each membrane and secondary antibodies were treated for 1 h followed by 5 min washing with PBST (0.3% Tween-20). The detection of antibodies were done by ECL Kit using X-ray.

Transient transfection of GFP-LC3 in HMC3 cells:

HMC3 cells were seeded on c coverslips and were transfected with GFP-LC3 plasmid (1 µg of plasmid+ Lipfectamine 20 µl per ml) for 24 h. The transfected cells were treated with betaine at a concentration of 1, 5 and 10 mM. for 24 h. These cells were fixed for 30 min with 5% paraformaldehyde and slides were prepared by using mounting media and analyzed through confocal microscope.

Amyloid beta clearance through confocal microscope:

HMC3 cells were seeded into coverslips and pre-treated with betaine at a concentration dependent manner for 12 h followed by the treatment of hilyte Aβ₁₋₄₂ 555 for 24 h and DAPI (1 mg/ml) for 15 min. Cells were fixed with 5% paraformaldehyde for 30 min and slides were prepared for confocal microscopy.

ELISA for amyloid beta clearance:

HMC3 cells were pre-treated with betaine at a concentration dependent manner for 12 hours and Ab₁₋₄₂ for 24 h. Cells were washed, lysed and performed as suggested by the manufacturer instructions.

Transient transfection of LC3 in differentiated SHSY5Y cells:

SHSY5Y cells were differentiated into retinoic acid for 7 days and transfected with siLC3 for 24 h. After 24 h, cells were pre-treated with betaine followed by Ab₁₋₄₂ for 48 h. Before 4 h of termination MTT were added followed by DMSO into each well. The absorbance was taken at 570 nm on plate reader. The untreated controls were taken as 100% and the comparison were made between control vs amyloid beta and amyloid beta vs control.

Neuroprotection:

Differentiation of human SHSY-5Y cells was obtained by 5% FBS and 10 mM retinoic acid for 7 days. After differentiation, betaine was pre-treated into SHSY-5Y for 24 h and Ab₁₋₄₂ (10 mM) treatment for 48

hours. MTT dye (2.5 mg/ml) was incubated into each well. After 4h, DMSO was added into each well. The absorbance was taken at 570 on plate reader. The untreated controls were taken as 100% and the treated groups were compared with control.

Statistical Analysis:

Mean $\pm$ SD of three independent experiments is shown here and students T-test were performed for comparison between groups. $P < 0.05$ was considered as statistical significant.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

Qingmian Meng wrote, designed and performed most of the experiments. Yan Chen and Ying Chen contributed to the repetition of the experiments; Lijun Wang performed the neuroprotection assay. Lan Zhao provides help with confocal microscopy. All authors read and approved the final manuscript.

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Figures

Figure 1

Betaine induced autophagy in HMC3 cells in a concentration dependent manner. (A) Western blots analysis showed the conversion of LC3-I into LC3-II into HMC3 cells in a concentration dependent manner. (B) Histograms represents the densitometry of western blots (C) Confocal microscopy indicates LC3 puncta in HMC3 cells treated with betaine. (D) Quantification of LC3 puncta was calculated by ImageJ software of three independent experiments. Statistical comparisons were made between the betaine treated samples vs untreated samples by using ANOVA followed by Bonferroni method. The p value <0.05 was considered to be significant. p value * <0.5 , ** <0.01 , *** <0.001 .

Figure 2

Figure 2

Inhibition of PI3K/AKT pathway induced autophagy by betaine in HMC3 cells. (A) Western blot analysis showed the decreased expression of p-AKT (ser 473), p-MTOR (S2448) and activation of ATG5, ATG7, ATG12 and beclin in HMC3 cells treated with betaine. (B) Quantification of western blot was analyzed by ImageJ software. Statistical comparisons were made between the betaine treated samples vs untreated samples by using ANOVA followed by Bonferroni method. The p value <0.05 was considered to be significant. p value * < 0.05, ** < 0.01, *** < 0.001.

Figure 3

Amyloid beta clearance by betaine in HMC3 cells. Confocal microscopy of HMC3 cells depicts the clearance of amyloid beta as the fluorescence of hilyte A β 1-42 555 was decreased in a concentration dependent manner. Statistical comparisons were made between the betaine treated samples vs untreated samples by using ANOVA followed by Bonferroni method. The p value <0.05 was considered to be significant. p value *< 0.5, **<0.01, ***<0.001.

Figure 4

Figure 4

Betaine protects differentiated SHSY-5Y cells in a concentration dependent manner. Amyloid beta induced toxicity was rescued by betaine in a concentration dependent manner. Data represented here are the mean $\pm$ standard deviation of three independent experiments. Bonferroni's method was used for Statistical comparisons. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs 0 μM groups.

Figure 5

Figure 5

Knockdown of LC3 hampers neuroprotection induced by betaine. The bar graph represented the reversal of neuroprotection provided by betaine in siLC3 SHSY5Y transfected cells in a concentration dependent manner. Statistical comparisons were made between the betaine treated samples vs untreated samples by using ANOVA followed by Bonferroni method. The p value <0.05 was considered to be significant. p value * < 0.5, ** < 0.01, *** < 0.001.