β-Hydroxybutyrate Ameliorates Aβ-Induced Downregulation of TrkA Expression by Inhibiting HDAC1/3 in SH-SY5Y Cells

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Abstract
Tyrosine kinase receptor A (TrkA) plays an important role in the protection of cholinergic neurons in Alzheimer’s disease (AD). This study was designed to investigate whether β-hydroxybutyrate (BHB), an endogenous histone deacetylase (HDAC) inhibitor, upregulates the expression of TrkA by affecting histone acetylation in SH-SY5Y cells treated with amyloid β-protein (Aβ). The results showed that BHB ameliorated the reduction of cell vitality and downregulation of TrkA expression induced by Aβ. Furthermore, BHB inhibited the upregulation of HDAC1/2/3 expression and downregulation of histone acetylation (Ace-H3K9 and Ace-H4K12) levels in Aβ-treated cells. The expression of TrkA was upregulated in HDAC1- or 3-silenced SH-SY5Y cells. However, there was no significant difference in TrkA expression between the HDAC2 knockdown and control cells. In conclusion, this study demonstrates that BHB protects against Aβ-induced neurotoxicity in SH-SY5Y cells. The underlying mechanism of the effect may be associated with the upregulation of TrkA expression by inhibiting HDAC1/3.

Keywords
Alzheimer’s disease, β-hydroxybutyrate, tyrosine kinase receptor A, histone deacetylases

Introduction
Alzheimer’s disease (AD) is a chronic neurodegenerative disease characterized by continuous exacerbation in cognitive, language, executive, and behavioral functions. Its pathological hallmarks are extracellular plaques and intracellular neurofilbrillary tangles, which consist of β-amyloid (Aβ) and hyperphosphorylated tau, respectively. Excessive accumulation of Aβ leads to the formation of toxic oligomers that destroy synapses and neurons. Accumulating evidence revealed that the progressive loss of memory and decline in cognitive function are due to the death of neurons, particularly cholinergic neurons, in brains affected by AD. The effective protection of cholinergic neurons is of great importance to the prevention and therapeutics of AD.

Nerve growth factor (NGF) is the first identified neurotrophic factor participating in neuronal differentiation, growth, survival, and functional maintenance. Nerve growth factor binds to 2 transmembrane receptors, high-affinity receptor tyrosine kinase receptor A (TrkA) and low-affinity receptor p75 neurotrophin receptor (p75NTR). The biological effects of NGF are mainly mediated by its high-affinity receptor TrkA, while TrkA expression is decreased in cholinergic neurons of the striatum and basal forebrain in patients with AD. The lack of neurotrophic support due to the reduction of TrkA may play an important role in cholinergic neurons death in patients with AD. Therefore, upregulating TrkA expression is crucial to protect cholinergic neurons in AD. It has been documented that TrkA expression is regulated by histone acetylation modification. Histone acetylation occurs at different lysine positions, especially histone 3 lysine 9 (H3K9) and histone 4 lysine 12 (H4K12) acetylations are implicated in cognition and...
Histone acetylation is modulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). There is increasing evidence that HDACs may be the potential targets for the treatment of AD. In mammals, HDACs are divided into 4 groups: zinc-dependent class I, II, and IV HDACs, and nicotinamide adenine dinucleotide-dependent class III HDACs (also known as sirtuins). Previous studies showed that the expression of TrkA is upregulated in both ΔNp73-overexpressed PC12 cells treated with a pan-HDAC inhibitor trichostatin A and traumatic brain injury in rodent models exposed to another pan-HDAC inhibitor LB-205. Iraci et al found that recruitment of class I HDAC1 induces a decrease in histone acetylation of the core promoters of TrkA in human TET-21/N cell line. However, it is still unclear whether HDAC1 plays a role in the regulation of TrkA transcription by altering histone acetylation status in AD. Moreover, class I HDAC2 and 3 are known to be strongly associated with AD. More importantly, it remains unknown whether HDAC2/3 participates in regulating the expression of TrkA in AD.

β-Hydroxybutyrate (BHB), a major component of the ketone bodies, is an endogenous HDAC inhibitor. In APP swe/PS1dE9 transgenic mouse model of AD, peripheral administration of BHB significantly reduces Aβ burden and greatly improves learning and memory ability. β-Hydroxybutyrate also protects the cultured hippocampal neurons from Aβ toxicity. However, the mechanism underlying the action of BHB against AD is not clarified. As an in vitro model, Aβ-treated SH-SY5Y cell line has been widely used to explore the mechanisms of action in AD. Therefore, the present study investigated the effects of BHB in SH-SY5Y cells exposed to Aβ. The results indicate that BHB upregulates TrkA expression by inhibiting HDAC1/3 to exert neuroprotective effects.

Materials and Methods

Reagents

β-Hydroxybutyrate was purchased from Shanghai Yingxin Laboratory Equipment Co, Ltd (Shanghai, China); all-trans retinoic acid (RA) was obtained from Sangon Biotech (Shanghai, China); rabbit anti-TrkA polyclonal antibodies (ab76291) were obtained from Abcam (Cambridge, UK); rabbit anti-Ace-H4K12 (06-1352) polyclonal antibodies were purchased from Santa Cruz Biotechnology (California, USA); rabbit anti-HDAC1 (06-1352), rabbit anti-HDAC2 (sc-7899), rabbit anti-HDAC3 (sc-11417), and rabbit anti-β-actin (sc-10731) polyclonal antibodies were purchased from Santa Cruz Biotechnology (California, USA); rabbit anti-Histone H3.1 polyclonal antibodies (18-785-210201) were obtained from GenWay Biotechnology Inc. (San Diego, USA). The secondary antibodies used were conjugated to horseradish peroxidase, goat polyclonal anti-rabbit (31460; Thermo Fisher Scientific, USA). Cell culture media were from American Hyclone Inc. (USA). Aβ25-35 (toxic effect fragment of an Aβ peptide) was from American Peptide Inc. (USA), which was solubilized in sterile water at 1 mmol/L concentration and aggregated by in vitro incubation at 37°C for 7 days.

Cell Culture

Human neuroblastoma SH-SY5Y cells (KCB2006107 YJ; Chinese Academy of Sciences cell bank, Kunming, China) were grown in a mixture (1:1, vol/vol) of Dulbecco modified Eagle medium (DMEM) and Ham’s F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified incubator containing 95% air and 5% CO2. All-trans RA was used to differentiate SHSY-5Y cells. After 24 hours of plating, cells were incubated in medium containing all-trans RA (final concentration of 10 μmol/L) for 3 days.

Cell Viability Assay

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, differentiated SH-SY5Y cells were seeded in 96-well culture microplates at a density of 8 × 103 cells/well. Cells were pretreated with or without BHB (at a final concentration of 0, 5, 10, 20, 40, or 80 mmol/L) for 24 hours. Then, a 10 μL solution of MTT (5 mg/mL) was added to each well and cells were continuously incubated for 3 hours. The purple MTT formazan crystals were subsequently dissolved by adding a 100 μL solution of dimethyl sulfoxide to each well. Absorbance was measured at 490 nm with Microplate Reader (Biotek, USA). The experiment was performed in duplicate and repeated 3 times. Cells were pretreated with or without BHB (at a final concentration of 5 mmol/L). After 3 hours, the cells were exposed to Aβ (at a final concentration of 0, 10, 20, 40, or 80 μmol/L) and were continuously incubated for 24 hours. Then, the cell viability was determined by the above-mentioned MTT assay.

Cells Treatment for Quantitative Real-Time-Polymerase Chain Reaction and Western Blot Analyses

Differentiated SH-SY5Y cells were seeded in 6-well culture microplates at a density of 1 × 105 cells/well in 2 mL of antibiotic-free normal growth medium and incubated for 24 hours. The experiment was designed into 4 groups: control, BHB, Aβ, and Aβ+BHB. Cells in BHB group and Aβ+BHB group were pretreated with BHB (at a final concentration of 5 mmol/L); cells in other groups were incubated in BHB-free medium. Three hours later, 40 μL Aβ25-35 (1 mmol/L) was mixed quickly into the 2 mL medium in Aβ group and Aβ+BHB group, which Aβ25-35 at a final concentration of 20 μmol/L (no effect on cell viability), continuing to incubate cells for 24 hours. The cells were collected and used for quantitative real-time-polymerase chain reaction (qRT-PCR) and Western blot analyses. The experiments were performed in duplicate and repeated 3 times.
Quantitative Real-Time-Polymerase chain Reaction Analysis

Total messenger RNA (mRNA) from the collected cells was isolated using Trizol (CWBO, Beijing, China). Reverse transcription and first-strand synthesis from each sample were carried out using the HiFiScript cDNA Synthesis Kit (CWBO). Resulting complementary DNAs were used as templates for qRT-PCR in the ABI 7500 Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Inc., Carlsbad, California) using Ultra SYBR Mixture (CWBO). The PCR reaction was performed as reported before.\(^{18}\) The primers were synthesized and purified by Shanghai Sangon Biotech with the following sequences: homo TrkA forward: 5'-AGGTTGAAGCATTCTCCTG-3', reverse: 5'-TTCGGTTGGAACCTAGG-3' (145 bp product); homo HDAC1 forward: 5'-ACUGGCGGATGGTGGAAAT-3', reverse: 5'-TGGTTTGTAGGCCCAGT-3' (135 bp product); homo HDAC2 forward: 5'-AGGGTGAAGCATTCTCCTG-3', reverse: 5'-ATCCGCACTTTGGAAGG-3' (179 bp product); homo HDAC3 forward: 5'-GGAGTTGAGGGTAACGCA-3', reverse: 5'-CAGGTTGAGGGTACGCA-3' (137 bp product); and β-actin forward: 5'-CATCCGTAAACAGCTCTATGGCACA-3', reverse: 5'-ATGGAGCCACCGTCCACA-3' (171 bp product). The PCR reaction was amplified in the program including an initial denaturation step at 95°C for 30 seconds, followed by 40 cycles for denaturation at 95°C for 5 seconds, annealing at 60°C for 34 seconds, subsequent melting curves for 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C to ensure that only a single product was amplified. Absolute values from each sample were normalized to β-actin (constitutive gene) mRNA as a reference standard.

Western Blot Analysis

The collected cells were directly homogenized in radioimmunoprecipitation assay buffer containing 0.1% protease inhibitor (Amersco, USA). The lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C, and the supernatants were used for protein analysis. The protein concentration was determined by the Bradford method with Coomassie Brilliant Blue (CBB G-250). Supernatants were mixed with β-mercaptoethanol (5%) and bromophenol blue (0.02%) and boiled for 5 minutes to denature the proteins. Equal amounts of soluble protein were separated by sodium dodecyl–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. After blocking with blocking buffer for 1 hour, the membrane was, respectively, incubated with rabbit anti-TrkA (1:2000), anti-HDAC1 (1:2000), anti-HDAC2 (1:2000), anti-HDAC3 (1:2000), anti-Ac-H3K9 (1:200), anti-Ac-H4K12 (1:200), anti-Histone H3.1 (1:200), and anti-β-actin (1:1000) polyclonal antibodies overnight at 4°C, followed by incubation with goat anti-rabbit immunoglobulin G secondary antibody (1:8000) for 2 hours. Immunoreactive protein bands were detected by Gel Image System Ver. 4.00 (USA). The bands were quantified using the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland). Variations in sample loading were normalized relative to β-actin or histone H3.1, which were used as reference standards.

Small-Interfering RNA Knockdown

HDAC1, 2, and 3 small-interfering RNA (siRNA) duplexes (Guangzhou RiboBio Co, Ltd, China) were used simultaneously (si-HDAC1-2-3) or singly (si-HDACs) to interfere with endogenous HDAC1, HDAC2, and HDAC3 expression. The siRNA oligos were as follows: HDAC1: 5'-CCGTCATGTCCAAAGTGAA-3'; HDAC2: 5'-TCCGTAATGTGGCTCGATG-3'; and HDAC3: 5'-GCATTGATCCAGAGTCTTA-3'. In brief, differentiated SH-SY5Y cells were seeded in 6-well culture microplates at a density of 1 × 10^5 cells/well in 2 mL of antibiotic-free normal growth medium. The following day, cells were transfected with a solution of 50 nmol/L siRNA duplex and 12 µL of siRNA transfection reagent (Guangzhou RiboBio Co, Ltd) mixed in DMEM/F12 medium containing 10% FBS. The transfected cells were incubated at 37°C for 24 hours. The untreated (non-siRNA) and nonspecific siRNA treated (scrambled siRNA; Guangzhou RiboBio Co, Ltd) cells were used as controls. The interference efficiency was evaluated by qRT-PCR and Western blot analyses. In addition, the expression levels of TrkA mRNA and protein were investigated by the above methods. This experiment was performed in duplicate and repeated 3 times.

Statistical Analysis

All data are presented as the means ± standard deviation. One-way analysis of variance followed by post hoc Fisher least significant difference was used for data analyses in this study. The statistical analysis was performed with SPSS version 20.0 software. Probability values of less than .05 were considered to be statistically significant.

Results

β-Hydroxybutyrate Ameliorated the Decrease of Cell Viability in Aβ-Treated SH-SY5Y Cells

The results indicated that the cell viability was markedly decreased in cells treated with 40 or 80 mmol/L BHB (Figure 1A), and with 40 or 80 µmol/L Aβ (Figure 1B). After BHB pretreatment, the cell viability was significantly restored in cells exposed to 40 or 80 µmol/L Aβ (Figure 1B).

β-Hydroxybutyrate Ameliorated the Decrease of TrkA Expression in Aβ-Treated SH-SY5Y Cells

As shown in Figure 2, the expression levels of TrkA mRNA and protein were markedly reduced in cells exposed to Aβ and significantly increased in BHB-treated cells. Compared with the cells only treated with Aβ, the expression levels of TrkA mRNA and protein were significantly increased in cells treated with both BHB and Aβ.
β-Hydroxybutyrate Alleviated the Decreases of Ace-H3k9 and Ace-H4k12 Levels and the Increase of HDAC1/2/3 Expression in Aβ-Treated SH-SY5Y Cells

The levels of Ace-H3K9 and Ace-H4K12 were both dramatically declined in Aβ-treated cells and significantly upregulated in cells with BHB treatment; BHB pretreatment significantly reversed the decreases of Ace-H3K9 and Ace-H4K12 levels induced by Aβ (Figure 3A-C). As illustrated in Figure 3D-F, the expression levels of HDAC1/2/3 mRNA and protein were greatly upregulated in cells exposed to Aβ and significantly downregulated in cells with BHB treatment; BHB pretreatment significantly reversed the upregulation of HDAC1/2/3 expression induced by Aβ.

Knockdown of HDAC1 or 3 But Not HDAC2 Upregulated the Expression of TrkA

The results revealed that si-HDAC1-2-3 significantly upregulated the expression levels of TrkA mRNA and protein in cells compared with controls (Figure 4A-C). Furthermore, singly knockdown of HDAC1 or 3 markedly elevated TrkA mRNA and protein expression levels in cells (Figure 4D-F and J-L) compared with controls; however, there was no obvious difference in TrkA expression between HDAC2 knockdown cells and controls (Figure 4G-I).

Discussion

β-Hydroxybutyrate is a product of the metabolism of fatty acid that accumulates during prolonged exercise, ketogenic diets, calorie restriction,14 acute fasting,15 and intermittent fasting.20 The concentration of BHB in blood can be increased to 1 to 2 mmol/L during fasting when the liver switches to fatty acid oxidation,19,21 and to even higher concentrations during prolonged fasting (6–8 mmol/L).22 It has been reported that BHB does not affect cell vitality at a concentration of 10 mmol/L in primary neurons.23 Imamura et al found that pretreatment with 8 mmol/L BHB has a significant neuroprotective effect on differentiated SH-SY5Y cells.24 The present study indicated...
that BHB, even at 20 mmol/L, had no effect on cell vitality in SH-SY5Y cells, and 5 mmol/L BHB pretreatment provided a significant protection against the reduction of cell vitality induced by Aβ, which is consistent with previous studies. Furthermore, 1 mmol/L BHB (a dose is available in physiological level) had the same effect (data not shown).

Nerve growth factor is an important player in synaptic plasticity and cognitive function, and TrkA is known to be the functional receptor of NGF. In patients with AD, TrkA expression is reduced in the basal forebrain nuclei, which may impair the NGF-initiated cell signal transduction and, ultimately, the cell survival. The data (not shown) from our study confirmed that the TrkA expression was decreased in the cerebral cortex and hippocampus of the AD model mice, and intermittent fasting, increase BHB level in blood, alleviated the decrease of TrkA expression. Moreover, in the present study, we demonstrated that BHB ameliorated the decrease of TrkA expression in SH-SY5Y cells treated with Aβ. Evidence showed that the SH-SY5Y cells can secrete neurotrophins such as NGF. Therefore, NGF/TrkA pathway may be involved in the protective actions of BHB against the reduction of cell viability induced by Aβ.

β-Hydroxybutyrate was reported to increase the expression of brain-derived neurotrophic factor (BDNF), a member of neurotrophic signaling, through inhibiting HDACs. Currently, it remains unknown whether the HDAC inhibitor BHB ameliorates Aβ-induced reduction of TrkA expression by modulating histone acetylation. The present study confirmed that BHB could reverse the upregulation of HDAC1/2/3 expression and the downregulation of histone acetylation levels induced by Aβ in SH-SY5Y cells. In addition, in AD model mice, the HDACs expression and histone acetylation levels regulated by intermittent fasting (increasing the blood BHB level) were same to results in Aβ-induced SH-SY5Y cells with BHB treated. A previous study showed that TrkA expression was repressed when HDAC1 bound to its promoter and restored when an HDAC inhibitor trichostatin A was used in neuroblastoma. This study also showed that the expression of TrkA was upregulated in HDAC1-silenced cells. Furthermore, there was an elevation in TrkA expression in HDAC3-silenced cells. However, no difference was observed in TrkA expression between HDAC2-silenced and scramble-treated cells. These findings indicate that the upregulation of TrkA expression by BHB is mediated by lowering HDAC1 and 3, at least partly, but not HDAC2. Further study is also necessary to find out whether the acetylation level on the promoter of TrkA can be regulated in HDAC1-or 3-silenced cells. Interestingly, we found that the HDAC1/2/3 simultaneous knockdown resulted in very similar effect on TrkA transcript levels in comparison to HDAC1- or HDAC3-silenced cells, suggesting that although HDAC1/3 levels are changed upon BHB treatment, they rather act through unknown element that directly regulates TrkA mRNA levels. It could be also helpful to study a dose-dependent response to BHB in our model.

There are still several limitations in this study: other mechanisms of action may also participate in the protection of BHB against AD, such as preventing oxidative stress, regulating metabolism, or BDNF pathway. In addition, the
Conclusions

Taken together, BHB possesses a protective effect against Aβ-induced neurotoxicity in SH-SY5Y cells. The mechanism underlying the effects may be associated with the upregulation of TrkA expression by inhibiting HDAC1/3. This study indicates that the endogenous HDAC inhibitor BHB has a potential application in AD therapeutics.

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Author Contributions

The authors Xinhui Li, PhD Candidate, ZhipengZhan, PhD, contribute equally to this work.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Supplemental Material**

Supplemental material for this article is available online.

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