D-beta-hydroxybutyrate exhibits protective effects against microglia activation in lipopolysaccharide-treated mice and BV-2 cells

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Abstract

Microglia activation is the key player in neuro-inflammation, which is the central process of neuro-disorders. The protective effects of D-beta-hydroxybutyrate (BHB) against microglia activation were evaluated in lipopolysaccharide (LPS)-treated mice and BV-2 cells. Behavioral test, morphological change and immunofluorescence of microglia marker ionizing calcium-binding adaptor molecule 1 (IBA-1) in the hippocampus, and the generation of inflammatory interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and protective brain-derived neurotrophic factor (BDNF) and transforming growth factor-β (TGF-β) in the brain, were measured in LPS and/or BHB treated mice. In addition, the effects of BHB on the generation of IL-6, TNF-α, BDNF and TGF-β, and reactive oxygen species (ROS) level were detected in LPS-stimulated BV-2 cells. We found that BHB treatments attenuated behavioral abnormality of LPS-treated mice and reduced the number of IBA-1-positive cells with attenuation of cell morphological changes in the hippocampus. Meanwhile, BHB inhibited IL-6 and TNF-α generations; but promoted BDNF and TGF-β generations in the brain in LPS-treated mice. Furthermore, BHB concentration-dependently inhibited IL-6 and TNF-α generations, promoted BDNF and TGF-β generations, and reduced the level of reactive oxygen species with attenuation of cellular changes in LPS-stimulated BV-2 cells. In conclusion, BHB exhibits protective effects against microglia activation in LPS-treated mice and BV-2 cells, antagonizing neuro-inflammation.

Introduction

Neuro-inflammation is generally believed to underlie the initiation and progression of numberous neuropathological diseases, such as Alzheimer's or Parkinson's disease, autism spectrum disorder, depressive disorder and so on (Siniscalco et al. 2018; Liu et al. 2019; Söderbom and Zeng 2020; Cao et al. 2022).

Neuro-inflammation is majorly regulated by brain resident macrophages, the microglia cells, which recognize and scavenge toxic components in the central nervous system (CNS) (Sánchez-Sarasúa et al. 2020; Saxena et al. 2021). Microglia can switch between M1 and M2 phenotypes, with distinguishable morphology and cytokine profile in different conditions (Sánchez-Sarasúa et al. 2020). In most settings, M2 phenotype is the resting and anti-inflammatory state to perform a surveillance role in healthy condition. And in M2 phenotype, microglia secretes protective factors, such as brain-derived neurotrophic factor (BDNF) and transforming growth factor (TGF) to play neuro-protective roles. However, the pathogenic factors, usually lipopolysaccharide (LPS), can activate microglia to switch to M1 phenotype, which secretes pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Sánchez-Sarasúa et al. 2020). Although the purpose of M1 phenotype is to neutralize and eliminate toxic molecules, persist microglia activation under pathological conditions causes a chronic inflammation process and neuropathological disorders (Saxena et al. 2021). Up to now, there still lacks effective therapy, and studies strive to search for treatments for microglia activation and neuro-inflammation (Gudoityte et al. 2021).
For decades, ketogenic diet, which may result in the increased production of ketone bodies in the liver, has been used for treatment of neuro-disorders, such as intractable epilepsy (Field et al. 2022; Kumar et al. 2022). Considering the limitations of ketogenic diet, more and more studies focus on ketone bodies (Kolb et al. 2021; Lilamand et al. 2021). Besides energy supply, the main ketone body D-beta-hydroxybutyrate (BHB) is found to act as a signaling molecular to exhibit various beneficial effects (Kolb et al. 2021; Li et al. 2021b; Yao et al. 2021). Our previous study proved several protective roles of BHB in cardiovascular system (Li et al. 2021a; Qi et al. 2021). However, the protective effects of BHB against microglia activation and neuro-inflammation in the CNS still need further clarification (Benito et al. 2020; Shippy et al. 2020; Huang et al. 2022).

In this study, after mice were treated with LPS and/or BHB, the behavioral and morphological changes was analyzed, the immunofluorescence of microglia marker ionizing calcium-binding adaptor molecule 1 (IBA-1) was illustrated, the generation of IL-6, TNF-α, BDNF and TGF-β in brain tissue was measured to investigate the protective effects of BHB against microglia activation in LPS-treated mice. In addition, the effects of BHB on the generation of IL-6, TNF-α, BDNF and TGF-β, and the level of reactive oxygen species (ROS), were ascertained in LPS-stimulated BV-2 cells.

Materials And Methods

Animal experiments

Fifty healthy male ICR mice (26.6 ± 1.6 g) were purchased from Charles River (Beijing, China) and maintained according to the procedures outlined by the Institutional Ethics Committee of Hebei Medical University. Mice were randomly divided into five groups: control (Con) group, high dose BHB (B2) group, LPS group, LPS + low dose BHB (LPS + B1) group, LPS + high dose BHB (LPS + B2) group. Considering neuro-disorders are usually accompanied with systemic inflammation (Siniscalco et al. 2018; Liu et al. 2019; Söderbom and Zeng 2020), intraperitoneal injection of LPS (1 mg/kg, Sigma; seven times on alternate days) was carried out to made neuro-inflammation mice model (Yang et al. 2020). Based on our previous study, high dose BHB (3 d/kg/day) was given in drinking water, and low dose BHB (300 mg/kg/day) was given by intragastrical administration (Qi et al. 2021; Yang et al. 2021; Huang et al. 2022). After completion of LPS treatment, behavioral test (open field test) was performed. At the end of the experiment, mice were killed and brain tissue was taken out for morphological analysis (HE staining), microglia activation detection (IBA-1 immunofluorescence), and the generation of inflammatory IL-6 and TNF-α, protective BDNF and TGF-β. The experiment procedure was shown in Fig. 1.

Open field test

Open field sociability test was performed as reported (Zhao et al. 2020). The tests were conducted during similar daytime with a 40 × 40 × 40 cm acrylic box. A 20 cm × 20 cm width square in the center was defined as the inner zone. After a 3 min preadaptation, mice were individually placed in the center of the cage and recorded for 5 min. Time spent in the inner zone and total distance traveled by mice were
recorded by video tracking system (SMART 3.0, Panlab, Spain). After each individual test, clean the instrument with 75% ethanol to avoid interference from the former mouse to the latter.

**Immunofluorescence**

The immunofluorescence of IBA-1 was detected according to the previous reports (Li et al. 2021a; Qi et al. 2021). The primary antibody was anti-IBA-1 (1:50 dilution; CatalogNo. GB12105; Servicebio Biology, Wuhan, China). The corresponding secondary antibodies were tetramethylrhodamine (TRITC)-conjugated (1:20 dilution; Catalog number 5230 – 0336; Seracare, Maryland, USA), with DAPI for nuclear staining. The image was observed under a fluorescence microscope (IX51; Olympus). The number of IBA-1 positive cells from three visual fields in each group was recorded and quantified with Image-Pro Plus 6.0 software.

**Cells and treatments**

Mouse microglia BV-2 cells were purchased from the Chinese Academy of Medical Sciences (Beijing, China), and cultured in DMEM containing 10% fetal bovine serum. After treated with different doses of LPS (0.5, 1 and 2 µg/mL) for 24 h, BV-2 cells were collected for measurement of the mRNA expression of *Il-6*, *Tnf-α*, *Bdnf* and *Tgf-β*, to determine the optimal concentration of LPS stimulation. Then the cells were treated with LPS (1 µg/mL) and LPS plus different concentrations of BHB (2, 4 and 8 mM) (Margolis and O’Fallon 2020; Qi et al. 2021). The mRNA expression and protein content of IL-6, TNF-α, BDNF and TGF-β, the ROS level and morphological change of BV-2 cells were measured.

**Quantitative real time RT-PCR**

The mRNA expression of *Il-6*, *Tnf-α*, *Bdnf* and *Tgf-β* was quantified by quantitative real time RT-PCR. Total RNA was isolated using Trizol reagent (Takara, Dalian, China) and reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Shanghai, China), followed by quantitative real time PCR amplification using specific primers (Supplemental Table 1).

**Western blotting**

The protein content of IL-6, TNF-α, BDNF and TGF-β was measured by western blotting, as previously reported (Li et al. 2021a; Qi et al. 2021). The antibodies used were: anti-IL-6 (1:200 dilution; catalogue no. A0286; ABclonal, Wuhan, China), anti-TNF-α (1:200 dilution; catalogue no. A0277; ABclonal), anti-BDNF (1:200 dilution; catalogue no. A1307; ABclonal), anti-TGF-β (1:200 dilution; catalogue no. A15103; ABclonal), and anti-β-actin (1:2000 dilution; catalogue no. D110001; Sangon Biotech, Shanghai, China). The band intensity was quantified (Quantity One v4.6.2 software, Bio-Rad) and calculated.

**ROS detection**

A detection kit (Catalogue no. Ros100; Zeta Life, Menlo Park, USA) was used to detect the ROS intensity in BV-2 cells. In general, the treated cells were incubated with 10 µM dichloro-dihydro-fluorescein diacetate for 20 min at 37°C in the dark. After three washes with PBS, the cells were collected for detection of the fluorescence intensity using a spectrofluorometer (SpectraMax i3; Molecular Devices, San Jose, CA, USA) with 490 nm excitation and 530 nm emission filters.
Statistical analysis

Data were presented as means ± SD. One-way ANOVA was used to analyse differences among groups, and differences between two groups were evaluated using the least significant difference test (two-tailed). Values of $p < 0.05$ were considered statistically significant.

Results

**BHB alleviated the behavioral abnormality of LPS-treated mice**

After LPS stimulation, the open field behavioral test was carried out. The time that mice spent in the inner zone was recorded and analyzed (Fig. 2A). Compared with that in the Con group, the time reduced in the LPS group ($p < 0.05$), but not changed in the B2 group. However, the time increased significantly in the LPS + B1 and LPS + B2 groups, compared with that in the LPS group ($p < 0.05$). In addition, the relative distance in the inner zone was recorded and analyzed (Fig. 2B). Similarly, the LPS-induced decrease in distance ($p < 0.05$), was significantly reversed in the LPS + B1/B2 groups ($p < 0.05$).

At the end of the experiment, mice were killed and brain tissue was taken out for HE staining. However, no distinguishable morphological change in the hippocampus was observed among the groups (Fig. 2C).

The results showed that BHB treatment alleviated the behavioral abnormality of LPS-treated mice, although BHB exhibited no effect on normal mice.

**BHB exhibited protective effects against microglia activation in LPS-treated mice**

The immunofluorescence of microglia marker IBA-1 was detected to reflect microglia activation in the hippocampus (Fig. 3A). Weak and scattered IBA-1 fluorescence with regular microglia morphology was seen in the Con and B2 groups. Bright IBA-1 fluorescence and expanded cell size with thick and short spikes were observed in the LPS group, indicating activated microglia. However, IBA-1 fluorescence and the microglia morphological changes were attenuated obviously in the LPS + B1/B2 groups, without distinctive difference between the two groups.

In addition, the number of IBA-1 positive cells was calculated (Fig. 3B). Compared with that in the Con group, the number of IBA-1 positive cells increased significantly in the LPS group ($p < 0.05$), without significant change in the B2 group. However, compared with the LPS group, the number reduced significantly in the LPS + B1/B2 groups ($p < 0.05$), consistent with the changes of IBA-1 fluorescence.

Furthermore, the mRNA expression of inflammatory *Il-6* and *Tnf-α*, and protective *Bdnf* and *Tgf-β* in the brain was evaluated (Fig. 4A). Compared with the Con group, *Il-6* and *Tnf-α* mRNA expressions increased; *Bdnf* and *Tgf-β* mRNA expressions decreased in the LPS group with significant difference ($p < 0.05$ or $p <$...
but no significant change was observed in the B2 group. The LPS-induced changes in their mRNA expressions were reversed by BHB treatments with statistical differences \((p < 0.05\) or \(p < 0.01\)). Then their protein contents were detected and calculated (Fig. 4B, C). Compared with the Con group, IL-6 and TNF-\(\alpha\) protein contents increased; BDNF and TGF-\(\beta\) protein contents decreased significantly in the LPS group \((p < 0.05\) or \(p < 0.01\)). But in accordance with their mRNA expressions, BHB treatments suppressed IL-6 and TNF-\(\alpha\) protein contents; elevated BDNF and TGF-\(\beta\) protein contents in LPS-treated mice \((p < 0.05\) or \(p < 0.01\)), indicating the protective effects of BHB against neuro-inflammation.

These results showed that either low or high dose BHB exhibited protective effects against microglia activation in LPS-treated mice.

**BHB inhibited inflammatory IL-6 and TNF-\(\alpha\); promoted protective BDNF and TGF-\(\beta\) in LPS-stimulated BV-2 cells**

At first, the mRNA expression of *Il-6, Tnf-\(\alpha, Bdnf* and *Tgf-\(\beta* was detected to determine the optimal concentration of LPS stimulation (Fig. 5). The results showed that 1 \(\mu\)g/mL LPS was sufficient to induce inflammatory factors *Il-6* and *Tnf-\(\alpha*, and suppress protective *Bdnf* and *Tgf-\(\beta* in BV-2 cells \((p < 0.05\) or \(p < 0.01\)). Based on, the effects of different concentrations of BHB (2, 4, 8 mM) on their mRNA expressions were evaluated (Fig. 6). Compared with the Con group, the mRNA expression of *Il-6* and *Tnf-\(\alpha* increased; *Bdnf* and *Tgf-\(\beta* decreased significantly in the LPS group \((p < 0.05\) or \(p < 0.01\)). Compared with the LPS group, the mRNA expression of *Il-6* and *Tnf-\(\alpha* decreased; *Bdnf* and *Tgf-\(\beta* increased for BHB treatments in a concentration-dependent manner, with significant difference for 4 and 8 mM BHB treatments \((p < 0.05\) or \(p < 0.01\)).

Meanwhile, the protein content of IL-6, TNF-\(\alpha, BDNF* and *TGF-\(\beta* was detected and calculated (Fig. 7). Compared with the Con group, IL-6 and TNF-\(\alpha* protein contents increased; BDNF and TGF-\(\beta* protein contents decreased significantly in the LPS group \((p < 0.05\) or \(p < 0.01\), revealing inflammatory activation of BV-2 cells. However, BHB treatments concentration-dependently suppressed IL-6 and TNF-\(\alpha* protein contents; elevated BDNF and TGF-\(\beta* protein contents, with significant difference for 4 and 8 mM BHB treatments \((p < 0.05\) or \(p < 0.01\)), indicating the protective effects of BHB against the inflammatory activation of LPS-stimulated BV-2 cells.

The results verified that 4 mM BHB was sufficient to inhibit inflammatory IL-6 and TNF-\(\alpha* generations; promote protective BDNF and TGF-\(\beta* generations, in LPS-stimulated BV-2 cells.

**BHB suppressed the ROS level in LPS-stimulated BV-2 cells**

Effects of BHB on the ROS level in LPS-stimulated BV-2 cells were analyzed (Fig. 8A). Compared with that in the Con group, the ROS intensity increased in LPS-stimulated BV-2 cells \((p < 0.01\). However, the LPS-induced increase in ROS intensity was significantly inhibited by 2, 4 and 8 mM BHB treatments \((p < 0.05\) or \(p < 0.01\), with 4 mM BHB exhibiting the better effect.
Then 4 mM BHB was used to observe the typical morphology of BV-2 cells (Fig. 8B). Relating to regular cell shape in the Con group, BV-2 cells became expanded with shorter and thick spikes for LPS stimulation. However, the LPS-stimulated cellular changes were obviously attenuated by BHB treatment, consistent with the changes of ROS level and inflammatory IL-6 and TNF-α generations.

The results manifested that BHB concentration-dependently suppressed ROS level and attenuated cellular changes of LPS-stimulated BV-2 cells.

**Discussion**

Although BHB may inhibit neuro-inflammation to play beneficial roles in CNS, the protective effects of BHB against microglia activation still need clarification. In this study, BHB is revealed to inhibit microglia activation, suppress inflammatory IL-6 and TNF-α, and promote protective BDNF and TGF-β in vivo and in vitro, attenuating behavioral abnormality of mice.

Microglia activation is the major contributor to neuro-inflammation and the related neuro-disorders (Saxena et al. 2021; McFarland and Chakrabarty 2022; Shao et al. 2022). Generally, microglia can be activated by LPS, which is also used in the study to make neuro-inflammation mice model (Yang et al. 2020; Wu et al. 2022a). Behavioral abnormality, activated microglia in the hippocampus, and increased generation of inflammatory IL-6 and TNF-α; decreased generation of protective BDNF and TGF-β in the brain, were observed in LPS-treated mice. These results confirmed that LPS induced microglia activation and behavioral abnormality of mice. Considering the central role of microglia activation in neuro-disorders, a growing number of studies have conducted to search for effective therapies against microglia activation and neuro-inflammation (Gudoityte et al. 2021; Medrano-Jiménez et al. 2022; Zhang et al. 2022).

Besides energy supply, ketone body BHB plays various protective roles in CNS as a signaling molecule (Poff et al. 2019; Pérez-Liébana et al. 2020; Wang et al. 2021; Yao et al. 2021). It’s reported that BHB has the ability to inhibit the heat stress-induced neuro-inflammation (Huang et al. 2022). And BHB could also ameliorate inflammation after spinal cord injury and inhibits inflammasome activation in Alzheimer’s disease (Shippy et al. 2020; Wu et al. 2020; Kong et al. 2021). In this study, BHB is verified to inhibit microglia activation in the hippocampus and attenuate the behavioral abnormality of LPS-treated mice. However, BHB exhibited little effect on the indicators of normal mice, indicating its specific protective effects against the LPS-stimulated microglia activation. Overall, no better effect of high dose BHB (3 d/kg/day) was observed than that of low dose BHB (300 mg/kg/day) in LPS-treated mice, suggesting proper dosage selection for BHB in clinical application.

A series of studies have been conducted to investigate the underlying mechanism of BHB’s beneficial roles (Wu et al. 2020; Li et al. 2021b; Wang et al. 2021). Generally, BHB acts as an inhibitor of histone deacetylase to elevate histone acetylation and promote transcription of protective genes (Li et al. 2021a; Ji et al. 2022; Wu et al. 2022b). For example, the neuro-protective BDNF has been proved to be up-regulated by BHB (Hu et al. 2020; Norgren et al. 2021). In the study, BHB was also found to promote the
generation of protective BDNF and TGF-β in the brain of LPS-treated mice and the LPS-stimulated BV-2 cells.

For suppression of neuro-inflammation, BHB is reported to inhibit NLRP3 inflammasome or pro-inflammatory signaling, such as NF-κB pathways (Shippy et al. 2020; Kong et al. 2021; Huang et al. 2022). However, there exits inconsistency about the effects of BHB on inflammatory factors (Fu et al. 2015; Benito et al. 2020). In this study, BHB was found to suppress the generation of inflammatory IL-6 and TNF-α in the brain in LPS-treated mice. Besides, BHB concentration-dependently suppressed IL-6 and TNF-α generations, and reduced the ROS level with attenuation of cellular changes in LPS-stimulated BV-2 cells, with 4 mM BHB exhibiting the most dominant effects. Collaborating with other reports (Qi et al. 2021; Huang et al. 2022), the results suggested that BHB with proper physiological concentration may be sufficient and better for its protective effects. However, the intrinsic suppression mechanism of LPS-stimulated IL-6 and TNF-α by BHB still needs further clarification. Probably, BHB up-regulates some core protective controllers against microglia activation, such as Nrf2 and Sirtuins (Fernando and Wijayasinghe 2021; Saha et al. 2022).

Overall, these results demonstrated that BHB inhibited the LPS-stimulated microglia activation, suppressed inflammatory IL-6 and TNF-α, and meanwhile, promoted protective BDNF and TGF-β, antagonizing neuro-inflammation and playing protective roles. However, in view of the various cell types in the brain, the results limiting to microglia, might partially explain the protective effects of BHB in the CNS.

In conclusion, BHB exhibited protective effects against microglia activation in LPS-treated mice and BV-2 cells, antagonizing neuro-inflammation and the behavioral abnormality of mice.

**Declarations**

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**Author contributions**

Yanning Li designed the study, reviewed and edited the manuscript. Yuping Zhang and Kun Liu performed experiments and analyzed the data. Yunpeng Li and Yujie Ma performed experiments and wrote the manuscript. Ting Wang, Yu Wang and Zihan Fan performed experiments. Jinsheng Qi discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

**Data availability**
The article contains all essential data. All the data and material from this study are available upon reasonable request through the corresponding author.

**Ethics approval** Ethical approval was given by the Institutional Ethics Committee of Hebei Medical University.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare that there is no conflict of interest associated with this manuscript.

**References**

1. Syed N (2020) β-Hydroxybutyrate oxidation promotes the accumulation of immunometabolites in activated microglia cells. Metabolites 10(9):346


6. Lu X (2020) Beta-hydroxybutyrate enhances BDNF expression by increasing H3K4me3 and decreasing H2AK119ub in hippocampal neurons. Front Neurosci 14:591177


15. Sánchez Ortiz M (2022) Efficacy and safety of ketone supplementation or ketogenic diets for Alzheimer's disease: a mini review. Front Nutr 8:807970Paquet C (DiValentin E)


20. Kivipelto M (2021) Serum proBDNF is associated with changes in the ketone body β-hydroxybutyrate and shows superior repeatability over mature BDNF: secondary outcomes from a cross-over trial in healthy older adults. Front Aging Neurosci 13:716594Sandebring-Matton A


36. Li Z (2021) β-Hydroxybutyrate alleviates learning and memory impairment through the SIRT1 pathway in D-Galactose-injured mice. Front Pharmacol 12:751028

**Figures**

Figure 1
Experiment arrangement. Mice received intraperitoneal injection of LPS for 7 times, and oral BHB treatments for continuous 19 days. After LPS administration, behavioral test was carried out. At the end of the experiment, mice were killed and whole brains were collected for further analysis.

Figure 2

Effects of BHB on the behavioral and morphological changes in LPS-treated mice. In open field test, the time that the mice spent in the inner zones (A) and the relative distance in the inner zone (B) were shown.
(n = 6). Data were means ± SD. * p < 0.05 versus Con; # p < 0.05 versus LPS; analysed by one-way ANOVA and least significant difference test. (C) Morphological change in the hippocampus was illustrated by HE staining. Scale bar, 100 μm.

**Figure 3**

Effects of BHB on microglia activation in the hippocampus of LPS-treated mice. (A) Immunofluorescence of microglial marker IBA-1 (red) was shown to reflect microglia activation. Scale bar, 100 μm. (B) The number of IBA-1 positive cells were calculated (n = 3). Data were means ± SD. * p < 0.05 versus Con; # p < 0.05 versus LPS; analysed by one-way ANOVA and least significant difference test.

**Figure 4**

Effects of BHB on the generation of IL-6, TNF-α, BDNF and TGF-β in the brain in LPS-treated mice. (A) The mRNA expression of *Il-6*, *Tnf-a*, *Bdnf* and *Tgf-β* was measured. The protein content of IL-6, TNF-α, BDNF and TGF-β was detected (B) and calculated (C). Data were means ± SD (n = 3). * p < 0.05 or ** p < 0.01, versus Con; # p < 0.05 or ## p < 0.01, versus LPS; analysed by one-way ANOVA and least significant difference test.

**Figure 5**

Effects of LPS on the mRNA expression of *Il-6*, *Tnf-a*, *Bdnf* and *Tgf-β* in BV-2 cells. For different concentrations of LPS stimulation (0.5, 1, 2 μg/mL), the mRNA expression of *Il-6*, *Tnf-a*, *Bdnf* and *Tgf-β* was detected. Data were means ± SD (n = 3). * p < 0.05 or ** p < 0.01, vs Con group; analysed by one-way ANOVA and least significant difference test.

**Figure 6**

Effects of BHB on the mRNA expression of *Il-6*, *Tnf-a*, *Bdnf* and *Tgf-β* in LPS-stimulated BV-2 cells. Effects of different concentrations of BHB (2, 4, 8 mM) on the LPS-stimulated expression of these genes. Data
were means ± SD (n = 3). * $p < 0.05$ or ** $p < 0.01$, vs Con group; # $p < 0.05$ or ## $p < 0.01$, vs LPS group; analysed by one-way ANOVA and least significant difference test.

**Figure 7**

Effects of BHB on the protein content of IL-6, TNF-α, BDNF and TGF-β in LPS-stimulated BV-2 cells. For different concentrations (2, 4, 8 mM) of BHB treatments, the protein content of IL-6, TNF-α, BDNF and TGF-β was detected (A) and calculated (B). Data were means ± SD (n = 3). * $p < 0.05$ or ** $p < 0.01$, vs Con group; # $p < 0.05$ or ## $p < 0.01$, vs LPS group; analysed by one-way ANOVA and least significant difference test.
Figure 8

Effects of BHB on the ROS level and morphological changes of LPS-stimulated BV-2 cells. (A) The ROS intensity for different concentrations (2, 4, 8 mM) of BHB treatments was measured (n = 3). Data were means ± SD. ** p < 0.01 versus Con; # p < 0.05 or ## p < 0.01 versus LPS; analysed by one-way ANOVA and least significant difference test. (B) The typical morphology of treated BV-2 cells was shown. Scale bar, 20 μm.
Supplementary Files

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- SupplementalTable1.doc