

Original Article

Metformin inhibits LPS-stimulated microglial proliferation and reduces the release of inflammatory cytokines

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Abstract: This study was designed to determine the effect of metformin on the release of inflammatory factors by lipopolysaccharide (LPS)-stimulated microglia and investigate the mechanisms potentially underlying these effects. The cytotoxicity of metformin was evaluated in microglia by trypan blue staining. Microglial cells were randomly divided into a control group, an LPS-stimulated group (LPS group: cells were cultured with 50 ng/mL LPS for 24 h) and a metformin plus LPS group (observation group: cells were first cultured with 0.02, 2, 8, or 16 mmol/L metformin for 24 h and then cultured with 50 ng/mL LPS for 24 h). TNF- α levels were measured by ELISA. Cell proliferation was evaluated by cell counting. Cell viability was evaluated by MTT assays. The cell cycle was examined by propidium iodide staining + flow cytometry. The apoptotic rate was analyzed by an improved propidium iodide staining + flow cytometry method. There were no differences in trypan blue staining among microglia treated with different concentrations of metformin. TNF- α levels were lower, proliferation was slower, and the apoptotic rate was higher in the metformin pretreatment group than in the group treated with only LPS, and these effects were dose-dependent. As the metformin concentration increased, the percentage of microglia in the G1 phase increased. There were significant differences in the percentage of microglia in the G1 phase between the LPS group and the observation groups pretreated with 8 and 16 mmol/L metformin. Metformin decreased the release of inflammatory factors from LPS-stimulated microglia, and this effect may be related to the reduced proliferation and increased apoptosis observed in the microglia.

Keywords: Metformin, microglia, lipopolysaccharide, tumor necrosis factor, cell cycle, apoptosis

Introduction

Microglia are the major immune protective cells in the central nervous system (CNS), and they closely reflect changes in the microenvironment in the CNS [1]. Under normal physiological conditions, microglia are in a resting state and have small cell bodies, few protrusions and some migratory capacity, but they do not perform phagocytosis, which can result in the secretion of growth factors to maintain the growth and differentiation of neurons [2]. Various sources of damage to the CNS can activate microglia and thereby contribute to the formation of amoebae. Microglia activation is a progressive process that initially manifests

as a neuroprotective effect; however, in long-term chronic inflammation, activated microglia produce a large number of proinflammatory cytokines (such as TNF- α) and superoxide compounds, both of which are neurotoxic. The accumulation of these substances leads to degeneration, deformation and even death in neurons and is one of the pathological mechanisms known to underlie the development of many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [3]. Therefore, inhibiting microglial activation and reducing the release of inflammatory cytokines may be an effective approach to treating neurodegeneration.

Antiinflammation effect of metformin

Since the 1950s, Metformin has been widely used in the treatment of type 2 diabetes, in which it reduces blood glucose levels by increasing insulin receptor sensitivity [4]. In recent years, many clinical and basic studies have found that metformin exerts anti-inflammatory effects in the peripheral and central nervous systems [5]. Most studies suggest that the anti-inflammatory mechanism involved in this effect is related to the activation of AMPK, the inhibition of the nuclear translocation of NF- κ B and subsequent reductions in the release of inflammatory factors [6, 7]. A chronic inflammatory reaction is an important pathogenic factor in neurodegenerative diseases, suggesting that metformin may have a therapeutic effect in neurodegenerative diseases. In some oncological diseases, metformin inhibits tumor cell proliferation and promotes apoptosis [8]. No previous study has explored the effect of metformin on microglial proliferation. The present experiments evaluated the effect of metformin on lipopolysaccharide (LPS)-treated microglial cells and include a preliminary study aimed at exploring the mechanism underlying this effect.

Methods

Cell culture

A mouse microglial cell line (BV-2) was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in DMEM (Thermo Fisher, Shanghai, China) supplemented with 10% fetal bovine serum (Sijiqing Bioengineering Materials, Hangzhou, China), 100 U/mL penicillin and 100 g/mL streptomycin (Thermo Fisher, Shanghai, China) in a culture incubator containing 95% air and 5% CO₂ (Thermo Fisher, Shanghai, China) at 37°C. The culture medium was replaced once a day. When cell growth was at approximately 80%, we observed the cultures under an inverted microscope (Olympus, Tokyo, Japan), and the cells were detached with 0.25% trypsin (Thermo Fisher Scientific Inc., Shanghai, China) for subculture.

Observation of metformin cytotoxicity

Cell membrane integrity was analyzed using trypan blue assays (Beyotime® Biotechnology, Jiangsu, China). BV-2 cells in the logarithmic

growth phase were inoculated into 6-well plates at a density of 1×10^5 cells/well. After pretreatment with 0.02, 2, 8, or 16 mmol/L metformin (Sigma-Aldrich, St. Louis, USA) for 24 h, the cell samples were centrifuged at 1000 rpm for 5 min. Then, the supernatant was discarded, and a 1×10^5 /mL single-cell suspension was obtained by adding a phosphate-buffered solution (PBS) (Thermo Fisher Scientific Inc., Shanghai, China). A 500 μ L volume of the cell suspension was added to 500 μ L of a 0.4% trypan blue working solution, and the mixture was observed under an inverted microscope (Canon, Tokyo, Japan) within 2 min. This dye stains dead cells blue because of the loss of cell membrane integrity and does not stain living cells.

Determination of BV-2 cell activity after metformin treatment

The MTT method (Beyotime® Biotechnology, Jiangsu, China) was used. BV-2 cells in the control group (blank) and the metformin treatment groups (0.02, 2, 8, and 16 mmol/L) were collected, and 10 μ L of 5 mg/mL MTT solution was added to each well of the plate, which was subsequently incubated for 4 h. After the cell supernatant was removed by a pipette, 150 μ L of DMSO (USEN Biological Technology, Shanghai, China) was added to each well. The mixture was shaken in the dark on an oscillator at room temperature until the particles were solubilized. Absorbance was measured at 490 nm in each well using an automatic microplate reader (BioTek Instrument, Winooski, USA) to evaluate cell viability.

Inhibition of BV-2 cell proliferation after metformin treatment

Cell proliferation was analyzed in the control group (blank) and in BV-2 cells that were treated with 0.02, 2, 8, and 16 mmol/L metformin. The rate of inhibition of cell proliferation was calculated as follows: [(cell number after metformin treatment - number of cells at seed plating) \div (cell number in the blank control - cell number at seed plating)] $\times 100\%$.

Cell grouping and processing

BV-2 cells in the logarithmic growth phase were seeded into 6-well plates at 1×10^5 cells/well and then randomly divided into a control group,

Antiinflammation effect of metformin

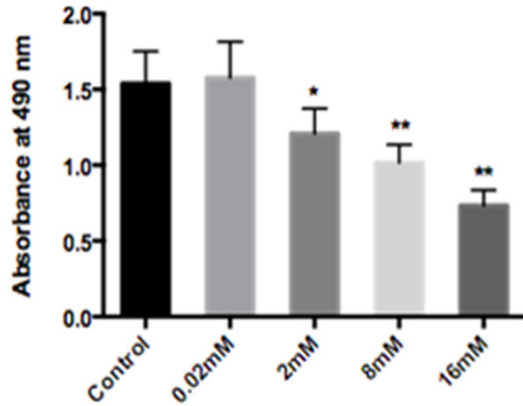


Figure 1. Effects of metformin on MTT values in BV-2 cells (compared with the control group: * $P < 0.05$, ** $P < 0.01$). (MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; mM: mmol/L).

an LPS group and an observation group. The control group was a blank control. The LPS group was treated with 50 ng/mL LPS (Sigma-Aldrich, St. Louis, USA) for 24 h. The observation group was pretreated with 0.02, 2, 8, or 16 mmol/L metformin for 24 h and then stimulated with 50 ng/mL LPS for 24 h.

BV-2 cell counts after metformin pretreatment and LPS post-processing

Cells in the LPS and observation groups (pretreated with metformin and then stimulated with LPS) were collected, and an equal volume of 0.4% trypan blue (Beyotime® Biotechnology, Jiangsu, China) was added to each sample. The cells were counted under an optical microscope (Olympus, Tokyo, Japan) within 2 min of the dye being added. The number of proliferating cells was calculated as follows: number of cells after treatment - number of cells when plated.

Detection of TNF- α in the supernatant of BV-2 cells

The ELISA method (Pusheng Biotechnology, Shanghai, China) was used. BV-2 cells were inoculated in 24-well plates at a density of 1×10^5 cells/well according to the groupings and processing described above (i.e., control, LPS and observation groups). The supernatants of the cells were collected and strictly processed according to the instructions included in a TNF- α kit. Finally, the absorbance was measured at 450 nm by a microplate reader (BioTek Instrument, Winooski, USA) to determine the TNF- α level.

Detection of cell cycle phases and apoptosis in BV-2 cells

Propidium iodide (Sigma-Aldrich, St. Louis, USA) staining and flow cytometry (Becton Dickinson, New York, USA) were used. Cells were collected from two groups (the LPS and observation groups) and washed with PBS 2 times. The cells were suspended in 500 μ L of DMEM and then fixed by adding 2 mL of 70% ethanol that had been precooled to -20°C . We were careful to ensure that the cells were shaken gently as the ethanol was added to prevent cell adhesion. Next, the cell samples were stored at -20°C for one night and then washed with PBS 2 times. DNA extraction solution (1 mL of a 192 mL solution containing 0.2 mol/L Na_2HPO_4 mixed with 8 mL of 0.1 mol/L citric acid with the pH adjusted to 7.8) (Tianhang Biotechnology, Zhejiang, China) was added to the cell sediment, and the mixture was placed on ice for 30 min. The mixture was then centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. Then, 400 μ L of PBS and RNA enzyme (100 μ g/mL) (Sigma-Aldrich, St. Louis, USA) was added to the cell pellet, and the mixture was incubated at 37°C for 30 min. Subsequently, the cell suspension was incubated with propidium iodide (50 μ g/mL) at 4°C for 30 min in the dark. The DNA content of each phase of the cells was determined by flow cytometry. A semiquantitative analysis of the cell cycle was performed using ModFit software (Verity Software House, Topsham, USA). The apoptotic rate was analyzed by an improved propidium iodide staining + flow cytometry method [9].

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, USA) was used for all statistical analyses. Descriptive results obtained using continuous variables are expressed as the mean \pm standard deviation. Intergroup comparisons were performed by one-way ANOVA and the Q test. Statistical significance was accepted at $P < 0.05$.

Results

Observation of metformin cytotoxicity

After BV-2 cells were treated with 0.02, 2, 8 or 16 mmol/L metformin for 24 h, the effects of metformin on the MTT values of BV-2 cells were

Antiinflammation effect of metformin

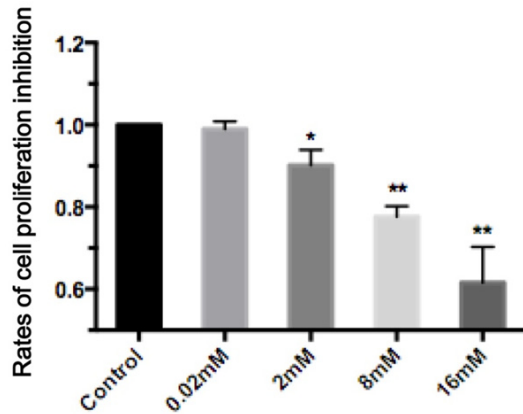


Figure 2. The effect of metformin on the inhibition of BV-2 cell proliferation (compared with the control group: * $P < 0.05$, ** $P < 0.01$). (mM: mmol/L).

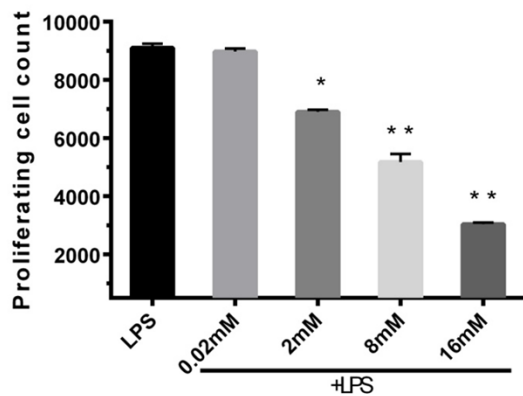


Figure 3. The effect of metformin on proliferating cell counts (compared with the LPS group: * $P < 0.05$, ** $P < 0.01$). (LPS: lipopolysaccharide; mM: mmol/L).

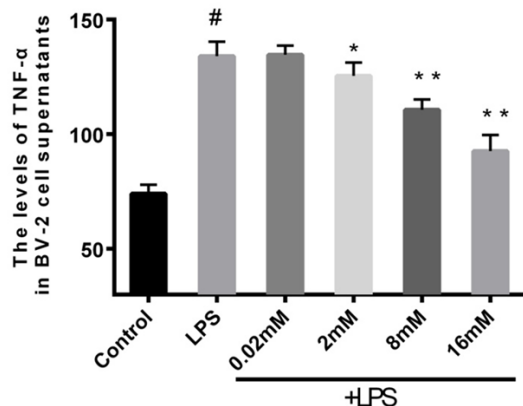


Figure 4. The effects of metformin on the release of TNF- α from BV-2 cells (compared with the control group: # $P < 0.01$; compared with the LPS group: * $P < 0.05$, ** $P < 0.01$). (LPS: lipopolysaccharide; mM: mmol/L).

observed. The absorbance values, which indicate proliferative activity, in these groups of BV-2 cells were 1.577 ± 0.281 , 1.210 ± 0.237 , 1.018 ± 0.186 , and 0.734 ± 0.143 , respectively, while the value in the control group (blank) was 1.539 ± 0.274 (Figure 1). At 24 h after metformin pretreatment, the MTT values were significantly lower in cells treated with higher concentrations of metformin, and these differences were significant for the cells treated with 2, 8 and 16 mmol/L metformin ($P < 0.05$, 0.01, and 0.01, respectively). However, no cells were clearly stained with trypan blue, suggesting a lack of clear drug cytotoxicity. The decreases observed in MTT values may have been caused by the metformin-induced inhibition of microglial proliferation.

Effect of metformin on the inhibition of BV-2 cell proliferation

As shown in Figure 2, compared to the control (blank) group, the metformin-treated groups (2, 8, and 16 mmol/L metformin) exhibited lower rates of cell proliferation inhibition in BV-2 cells, and these differences were statistically significant for the 2, 8, and 16 mmol/L metformin groups ($P < 0.05$, 0.01, and 0.01, respectively).

Effect of metformin pretreatment on BV-2 cell counts after LPS stimulation

As shown in Figure 3, the number of proliferating BV-2 cells counted in the observation groups that were pretreated with 0.02, 2, 8 and 16 mmol/L metformin and then stimulated with LPS were 8890 ± 440 , 6800 ± 405 , 5050 ± 420 , and 3060 ± 390 , respectively. The number of proliferating cells counted in the LPS group was 9000 ± 340 . The number of proliferating cells counted in the observation groups that were pretreated with 2, 8 and 16 mmol/L metformin were significantly lower than the number counted in the LPS group ($P < 0.05$, 0.01, and 0.01, respectively).

Effect of metformin on the release of TNF- α from BV-2 cells

As shown in Figure 4, the level of TNF- α in the BV-2 cell supernatants collected in the LPS group was 134.1 ± 0.78 ng/L, which was higher than the level observed in the control group ($P < 0.01$). The observation group was pretreated with 0.02, 2, 8, or 16 mmol/L metformin for

Antiinflammation effect of metformin

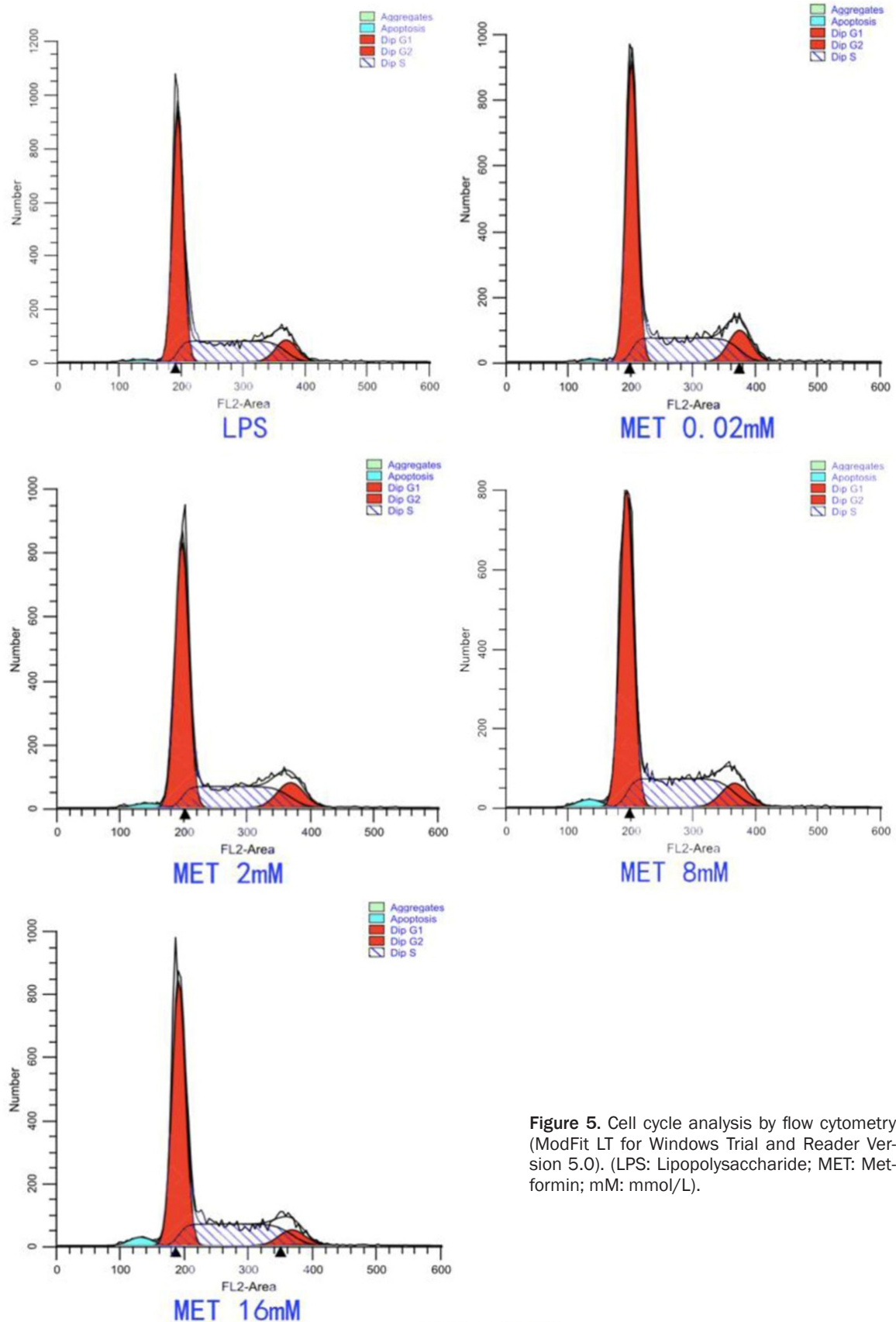


Figure 5. Cell cycle analysis by flow cytometry (ModFit LT for Windows Trial and Reader Version 5.0). (LPS: Lipopolysaccharide; MET: Metformin; mM: mmol/L).

Antiinflammation effect of metformin

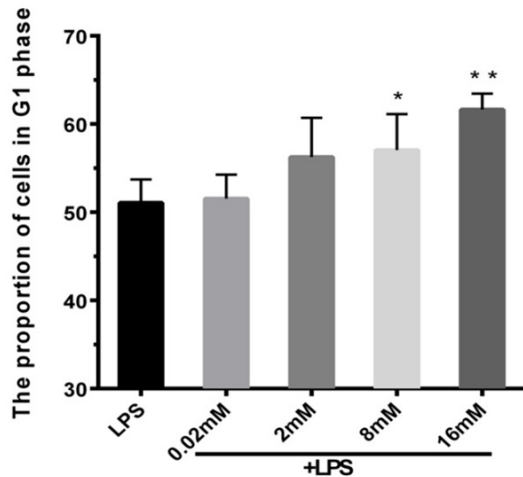


Figure 6. Effect of metformin on the cell cycle in BV-2 cells (compared with the LPS group: * $P < 0.05$, ** $P < 0.01$). (LPS: lipopolysaccharide; mM: mmol/L).

24 h and then stimulated with 50 ng/mL LPS for 24 h, and the TNF- α levels in the supernatants of these BV-2 cells were 134.60 ± 0.34 , 125.40 ± 0.48 , 110.70 ± 0.55 , and 92.69 ± 0.94 ng/L, respectively (ng/L). Compared with its release in the LPS group, the release of TNF- α in the observation group gradually decreased as the metformin concentration increased, and the differences were significant for cells exposed to metformin at 2, 8 and 16 mmol/L ($P < 0.05$, 0.01, and 0.01, respectively).

Effect of metformin on the cell cycle and apoptosis in BV-2 cells

In the observation group, the proportion of cells in the G1 phase gradually increased as the metformin concentration increased. Furthermore, the differences between the LPS group and the observation group were significant with the metformin concentration of 8 or 16 mmol/L ($P < 0.05$ and 0.01, respectively) (Figures 5 and 6). The apoptosis rate in the observation group gradually increased as the metformin concentration increased. Similarly, the differences between the LPS group and the observation group were significant for metformin concentrations of 2, 8 and 16 mmol/L ($P < 0.05$, 0.01, and 0.01, respectively) (Figures 7 and 8).

Discussion

CNS inflammation is a pathological feature of neurodegenerative diseases. During inflam-

mation, microglia become overactivated and release a large number of inflammatory factors that can damage neuronal cells and promote the progression of neurodegenerative diseases [10]. Recently, a large number of studies have shown that metformin exerts anti-inflammatory effects, and metformin is a candidate for exerting anti-inflammatory effects in the CNS because it was previously confirmed that metformin can pass the blood-brain barrier [11, 12]. Our results are consistent with those presented in previous studies that have shown that metformin reduces the endotoxin-induced release of TNF- α . In addition, in this study, we found that as the metformin concentration increased, its proliferation-inhibiting and apoptosis-promoting effects on microglia were enhanced. These two effects on microglia may represent mechanisms by which metformin could inhibit inflammation in the CNS.

In these experiments, we evaluated the effect of metformin on LPS-stimulated microglia. To exclude the possibility of metformin toxicity, which could interfere with the experimental results, we first carried out trypan blue staining. Different concentrations of metformin did not result in blue-dyed cells, confirming that the cell membrane was not damaged by metformin and excluding the possibility that the experimental results were caused by the toxicity of metformin itself. Additionally, we found that as the metformin concentration increased, the MTT values of microglia decreased, the inhibitory effect on cell proliferation increased, and the number of microglia arrested in the G1 phase increased. These results confirm that metformin inhibits microglial proliferation and that this inhibitory effect was not caused by metformin toxicity.

Some studies have found that the chronic inflammatory response in the CNS is related to the overexpression of cell cycle proteins in microglia and that inhibiting the microglial cell cycle exerts a neuroprotective effect [13, 14]. Gusain et al. [15] found that the sphingomyelinase inhibitor D609 blocked microglia in the G1 phase, thereby inhibiting microglial proliferation and playing a protective role in stroke. Varvel et al. [16] also found that non-catastrophic anti-inflammatory drugs, such as ibuprofen, stopped the cell cycle in microglia and promoted microglial apoptosis. Our results also show that metformin blocks the cell cycle in

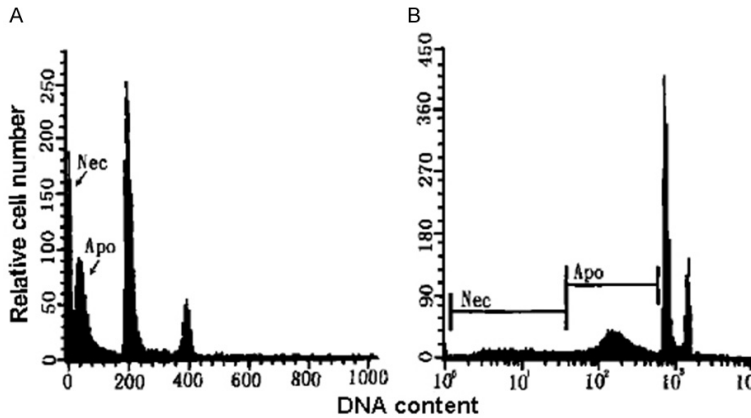


Figure 7. The “Sub-G1” method was used to measure cell apoptosis by propidium iodide staining and flow cytometry (Nec: Necrosis; Apo: Apoptosis).

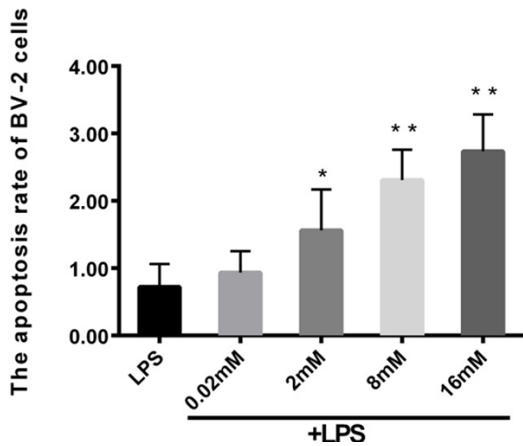


Figure 8. Effects of metformin on apoptosis in BV-2 cells (compared with the LPS group: *P<0.05, **P<0.01). (LPS: lipopolysaccharide; mM: mmol/L).

microglia in the G1 phase and inhibits microglial proliferation and the LPS-induced release of proinflammatory cytokines.

The cell cycle includes the G1, S, G2 and M phases; of these, the G1 phase is the only phase that can be stimulated by external factors. The transition at the restriction point during this phase determines whether cells continue to proliferate (enter the S phase), stagnate in the G0 phase, or undergo programmed death (apoptosis) [17]. We found that metformin blocked the cell cycle in microglia in the G1 phase, and we therefore further evaluated whether metformin induces apoptosis. In this study, we used a modified propidium iodide single-dye method to detect apoptosis. Propidium iodide staining is a classic method

of detecting apoptosis via flow cytometry. RNase is used to degrade RNA, allowing propidium iodide and the DNA in the cells to specifically bind to one another; the distribution of DNA is reflected in flow cytometry data. During apoptosis, endonucleases cleave DNA into many smaller fragments that then exit the cell when alcohol fixation is applied. This decreases the intracellular DNA content and reduces the amount of bound propidium iodide, resulting in the formation of a distribution region containing less DNA

than is observed in G1-phase cells. The resulting distribution region represents the apoptotic peak. However, when using this method, larger DNA fragments may not leak out of the cell, and the DNA content in the cell does not significantly decrease, preventing the apoptotic peak from being easily observed. The improved propidium iodide single-dye method more effectively extracts DNA from fixed cells, which have more permeable cell membranes, and increases the ability to extract fragmented DNA. Thus, intracellular small DNA fragments can more easily leave apoptotic cells, significantly improving the sensitivity of the method for identifying apoptotic cells [9]. We found that metformin pretreatment promoted LPS-induced microglial apoptosis in a concentration-dependent. Fujikawa R et al. [18] found that overactivated microglia release proinflammatory cytokines and other neurotoxic agents; hence, self-apoptosis may be a self-protection mechanism used by the body. Our results are consistent with previous findings that have suggested that promoting microglial apoptosis may represent a mechanism for suppressing the inflammatory response.

Conclusions

In summary, in this study, we found that metformin inhibited LPS-stimulated microglial proliferation and reduced the release of inflammatory cytokines. The mechanisms underlying these effects may be related to the ability of metformin to arrest the cell cycle and induce apoptosis.

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Disclosure of conflict of interest

None.

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