Early glycolytic reprogramming control microglial inflammatory activation

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Research

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Abstract

Background Microglial activation-mediated neuroinflammation plays an important role in the progression of neurodegenerative diseases. Inflammatory activation of microglial cells is often accompanied by a metabolic switch from oxidative phosphorylation to aerobic glycolysis. However, the roles and molecular mechanisms of glycolysis in microglial activation and neuroinflammation are not yet fully understood.

Methods The anti-inflammatory effects and its underlying mechanisms of glycolytic inhibition *in vitro* were examined in LPS activated BV-2 microglia or primary microglia cells by ELISA, RT-PCR, Western blot, immunoprecipitation, FACS and NF- κ B luciferase reporter assays. The anti-inflammatory and neuroprotective effects of glycolytic inhibitor, 2-DG *in vivo* were measured in the MPTP-or LPS-induced PD models by immunofluorescence staining, behavior tests and Western blot analysis.

Results We found that LPS rapidly increased glycolysis in microglial cells, and glycolysis inhibitors (2-DG and 3-BPA), siRNA Glut-1 and siRNA HK abolished LPS-induced microglial cell activation. Mechanistic studies demonstrated that glycolysis inhibitors significantly inhibited LPS-induced phosphorylation of mTOR, IKK β and I κ B, degradation of I κ B, nuclear translocation of p65 and NF- κ B transcriptional activity. In addition, 2-DG significantly inhibited LPS-induced acetylation of p65/RelA on lysine 310, which is mediated by NAD⁺-dependent SIRT1 and is critical for NF- κ B activation. A coculture study revealed that 2-DG reduced the cytotoxicity of activated microglia toward MES23.5 dopaminergic neuron cells with no direct protective effect. An *in vivo* study demonstrated that 2-DG significantly ameliorated neuroinflammation and subsequent DA neuronal cell injuries in an LPS-induced Parkinson's disease (PD) model. Furthermore, 2-DG also reduced TH-positive cell loss and microglial activation in the MPTP-induced PD model.

Conclusions Collectively, our results suggest that glycolysis is actively involved in microglial activation and, hence, that inhibition of glycolysis can ameliorate microglial activation-related neuroinflammatory diseases.

Introduction

Microglial cells are the resident immune effector cells of the central nervous system and are considered the cells critical for inflammation-associated neurotoxicity[1]. Under physiological conditions, microglial cells play critical roles in maintaining brain homeostasis through surveillance of the microenvironment of the brain with ramified morphology [1]. Under neurodegenerative conditions or upon experimental stimulation with lipopolysaccharide (LPS) or interferon (IFN)- γ , microglial cells become activated with amoeboid morphology [2]. Once activated, microglial cells produce a number of proinflammatory cytokines and factors, such as tumor necrosis factor (TNF)- α , nitric oxide (NO), prostaglandin (PG)E₂, interleukin (IL)-1 β , IL-6 and reactive oxygen species (ROS), that, in turn, contribute to the recruitment of other immune cells and neuronal cell injuries [2, 3]. There is growing evidence that uncontrolled activation of microglial cells is involved in the progression of several neurodegenerative diseases, including

Parkinson's disease (PD) [4]. Activated microglia-mediated neuroinflammation was found in both PD patients and rodent models of PD and was positively associated with dopaminergic neuron loss [4]. Therefore, inhibition of microglial inflammatory activation has become a potential therapeutic strategy against PD.

Glucose metabolism is actively implicated in microglia/macrophage activation-mediated inflammatory responses[5, 6]. The glucose catabolic metabolism pathway consists of glycolysis and mitochondrial oxidative phosphorylation. Glycolysis is defined as the breakdown of glucose through a series of extramitochondrial biochemical reactions into pyruvic and lactic acids under anaerobic conditions, which includes the glucose activation phase and the energy extraction phase [7]. Glycolytic flux is regulated by three rate-limiting steps that are mediated by three key enzymes: hexokinase, phosphofructokinase, and pyruvate kinase [7]. In the last step of glycolysis, pyruvate is converted to lactate, which is catalyzed by dehydrogenase (LDH) under anaerobic conditions [8]. Several lines of evidence have suggested that LPS-activated M1 macrophage cells display metabolic reprogramming, switching from oxidative phosphorylation to aerobic glycolysis, whereas M2 (anti-inflammatory) macrophage/microglial cells switch to oxidative phosphorylation [9]. LPS stimulation elicits enhanced aerobic glycolysis of macrophages upon the switch from L-PFK2 to uPFK2 expression [10]. Similar to macrophages or dendritic cells, activated M1-type microglial cells show a metabolic shift from oxidative phosphorylation to aerobic glycolysis [11].

Many studies have reported that inhibition of the glycolytic pathway can suppress inflammatory responses. Glucose transporter (Glut)1 was upregulated in activated microglial cells, and the inhibition of Glut-1 suppressed microglial activation [12]. Hexokinase II was induced in hypoxia-activated microglial cells, and the blockade of HK II suppressed ischemic brain injury by inhibiting microglia-mediated neuroinflammation [13]. 2-Deoxy-D-glucose (2-DG), a glucose analog, is phosphorylated by hexokinase and thereby competitively inhibits the production of glucose-6-phosphate from glucose and ultimately inhibits glycolysis [8]. In addition to its inhibitory effect on the proliferation of cancer cells, 2-DG has been recognized as a therapeutic agent for autoimmunity and inflammatory diseases [14]. The inhibition of glycolysis by 2-DG significantly reduced the production of some proinflammatory factors, such as HMGB and IL-1 β , whereas it did not affect other proinflammatory cytokines, including TNF- α [15, 16]. However, in LPS-activated dendritic cells, 2-DG treatment substantially decreased the production of proinflammatory cytokines such as IL-6, IL-12 and TNF- α only at translational levels [17]. Although the neuroprotective effects of 2-DG *in vitro* and *in vivo* have been demonstrated [18–20], the anti-neuroinflammatory activity of glycolysis inhibition has rarely been reported. Recently, we found that 2-DG inhibited the expression of TNF- α , IL-6, iNOS, COX-2 and IL-6 at the transcriptional level [21]. However, the underlying mechanisms by which 2-DG acts to mitigate the transcriptional expression of proinflammatory genes, particularly in microglial cells, are unclear. Furthermore, the effects of 2-DG on inflammation-mediated dopaminergic neuron cell loss have not yet been investigated. In the present study, we addressed the role of aerobic glycolysis in LPS-activated microglial cells and its potential significance in inflammation-induced dopaminergic neuronal cell death.

Methods And Materials

Materials

Bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 2-dexoy-D-glucose (2-DG) 3-bromopyruvic acid (3-BPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse TNF- α Quantikine ELISA kit and mouse IL-6 Quantikine ELISA kit were obtained from R&D Systems (Minneapolis, MN). Antibodies used in this study were as follows: anti-p-IKK α (S176)/IKK β (S177) (2078S), anti-IKK β (8943S), anti-p-IkBa (S32) (2859S), anti-IkBa (4814S), anti-NF- κ B p65 (8242S), anti-JNK (T183/Y185) (4668S), anti-JNK (9252S), anti-p-ERK 1/2 (9102S), anti-ERK 1/2 (4695S), anti-p-p38 (T180/Y182) (9215S), anti-p38 (9212S), anti-p-p70-S6K (T421/S132) (9204S), anti-p-mTOR (S2448) (2971S), anti-mTOR (2972S), and anti-p-AMPK (T172) (2535S) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-iNOS (ab15323), anti-NF- κ B p65 (acetyl K310) (ab19870) and anti- α -tubulin (ab7219) antibodies were from Abcam (Cambridge, MA, USA); and anti-Hexokinase β (HK2) antibody was from Bioword Technology (St. Louis Park, MN, USA). Anti-tyrosine hydroxylase (TH) was obtained from Millipore (Billerica, MA, USA), and anti-Iba1 was purchased from Wako Chemicals (Chuo-ku, Osaka, Japan).

Cell culture

The BV-2 murine microglial cell line and human embryonic kidney (HEK) 293T cell line were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, USA) containing 10% fetal bovine serum (FBS, PAN Biotech and Lonsera, Aidenbach, Germany), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco). MES23.5 dopaminergic cells require Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12, Gibco) with 5% fetal bovine serum (FBS, PAN Biotech and Lonsera), 100 U/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco) and 100x insulin-transferrin-selenium (Gibco). All three cell lines were cultured in an incubator at 37 °C in a 5% CO₂ atmosphere.

Primary culture of microglia

Primary cultures of microglia were collected from newborn C57BL/6J mice [22]. In summary, the newborn mice were washed in 75% alcohol, and the whole brains were isolated and minced in precooled PBS. Then, the cortical tissue was digested for 20 min with 0.25% trypsin. After centrifugation and resuspension, the samples were digested by DNaseI at 37 °C and transferred to a single cell suspension. Then, single cells were plated on poly-D-lysine-coated flasks for 14 days. The microglial cells were obtained from mixed glial cultures on a shaker at 180 rpm for 3 hours.

Nitric oxide (NO) measurement

The BV2 cell line was cultured in a 96-well plate at a density of 2.0×10^4 cells/well. After LPS or/and compound stimulation for 24 hours, 50 μ l of cell culture supernatant was transferred to a new 96-well plate and mixed with 50 μ l of Griess reagent. The signals were measured in a microplate reader (Infinite M200 PRO, Tecan, Switzerland) at 550 nm. The data were normalized to a standard curve.

Cytotoxicity assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, after appropriate treatment of BV2 cells, 30 μ l of MTT (Solarbio, Beijing, China) was added to each well and incubated at 37 °C for 4 hours. The samples were mixed with 100 μ l of DMSO and detected at 540 nm by a microplate reader (Infinite M200 PRO).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF- α and IL-6 in the medium were detected by mouse TNF- α or IL-6 ELISA kits according to the manufacturers' instructions.

RNA isolation and quantitative real-time PCR

Total RNA from the BV2 cell line or tissues was isolated by TRIzol reagent and subjected to reverse transcription by Oligo-d(T) and M-MLV reverse transcriptase (Thermo Fisher). Real-time quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR system (Foster City, CA, USA) using PrimeScript RT Master Mix (TaKaRa, Dalian, China). The specific primers used in reverse transcription were purchased from GENEWIZ (Suzhou, China) as shown in Table 1. Gapdh was used as a control. The normalized CT values were calculated according to the comparative delta-delta Ct method.

Table 1
primers used in real-time quantitative PCR

Primer	Sequence(5'-3')
mus-GAPDH	Forwad: TGTGTCCGTCGTGGATCTGA
	Reverse: TTGCTGTTGAAGTCGCAGGAG
mus-iNOS	Forwad: TAGGCAGAGATTGGAGGCCTTG
	Reverse: GGGTTGTTGCTGAACTTCCAGTC
mus-COX-2	Forwad: CAGGCTGAACTTCGAAACA
	Reverse: GCTCACGAGGCCACTGATACCTA
mus-TNF- α	Forwad: CAGGAGGGAGAACAGAAACTCCA
	Reverse: CCTGGTTGGCTGCTTGCTT
mus-IL-1 β	Forwad: TCCAGGATGAGGACATGAGCAC
	Reverse: GAACGTCACACACCAGCAGGTTA
mus-IL-6	Forwad: GCCAGAGTCCTTCAGAGAGA
	Reverse: GGTCTTGGTCCTTAGCCACT
mus-HK2	Forwad: TCATTGTTGGCACTGGAAGC
	Reverse: TTGCCAGGGTTGAGAGAGAG
mus-Glut-1	Forwad: CAGTTCGGCTATAACACTGGTG
	Reverse: GCCCCCGACAGAGAAGATG
mus-LDHA	Forwad: TGTCTCCAGCAAAGACTACTGT
	Reverse: GACTGTACTTGACAATGTTGGGA
mus-G6pdx	Forwad: CACAGTGGACGACATCCGAAA
	Reverse: AGCTACATAGGAATTACGGGCAA

Western blotting

BV-2 cells and 293T cells were lysed on ice for 30 min and shaken every 10 min in denaturing lysis buffer (50 mmol/L Tris [pH 8], 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mmol/L NaCl and PMSF), while mouse tissue (the substantia nigra and the striatum) was measured by an ultrasound system before being lysed on ice. After mixing with 5x loading buffer, the proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, followed by blocking in milk for 2 hours. Then, the samples were incubated with the appropriate primary antibodies and HRP-

conjugated secondary antibodies. Finally, proteins were detected by enhanced chemiluminescence (ECL) (Millipore) with a ChemiScope 3300 mini (CLINX, Shanghai, China).

Plasmids and siRNA transfection

BV-2 cells were seeded on a 12-well plate (5.0×10^4 cells/well) one day prior to transfection. the cells were transfected with appropriate siRNA or plasmids accompanied by transfection reagents (Lipofectamine®RNAiMAX or Lipofectamine 2000) according to the manufacturer’s instructions. After 24 hours, the cells were collected for subsequent experiments. The siRNA sequences are shown in Table 2.

Table 2
si-RNA used in RNA interference

Gene	siRNA	Sense (5'-3')
mus-HK2	siHK2	GGAGAUGCGUAAUGUGGAATT
mus-Glut-1	siGluT-1	GCUGCCUUGGAUGUCCUAUTT
mus-LDHA	siLDHA	CCACCAUGAUUAAGGGUCUTT
mus-G6pdx	siG6pdx	GGAGUUCUUUGCCCGUAAUTT

Coimmunoprecipitation (IP)

Protein was extracted in nondenaturing lysis buffer (20 mM Tris HCl [pH 8], 137 mM NaCl, 1% Nonidet P-40 (NP-40) and 2 mM EDTA), followed by incubation with appropriate antibodies overnight. Then, the samples were mixed with protein A/G beads for 4 hours, and then, the beads were washed 10 times to remove nonspecifically bound proteins. The beads and protein were mixed with 2x loading buffer and separated in boiling water, followed by Western blotting.

NF-κB luciferase reporter assays

BV-2 cells stably expressing the NF-κB reporter were seeded on a 12-well plate one day before compound and LPS (200 ng/ml) stimulation. After 16 hours, the cells were lysed in reporter lysis buffer, and luciferase activity was detected with a dual-luciferase assay kit following the manufacturer’s protocol (Promega, USA).

MPTP-induced PD model

All mice (male, C57BL/6J) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and placed in an SPF laboratory animal room. All the experiments used in this research followed the Guide for the Care and Use of Laboratory Animals (8th edition) and were approved by the Institutional Animal Care and Use Committee of Soochow University. In this experiment, mice were allocated to 4 groups: saline, 2-DG alone (400 mg/kg), MPTP alone (30 mg/kg) and 2-DG (400 mg/kg) + MPTP (30 mg/kg). For the 2DG + MPTP group, 2-DG was given 3 days prior to MPTP injections. MPTP was injected for 7 consecutive days, and 2-DG was preadministered 2 hours before MPTP injection. The

rotarod test and pole test were performed on the 11th day. After behavioral tests, mouse brain tissue was collected for further study.

LPS-induced PD model

Mice were separated into 4 groups: saline, 2-DG alone (400 mg/kg), LPS alone (5 mg/kg) and 2-DG (400 mg/kg) + LPS (5 mg/kg). For the 2DG + LPS group, 2-DG was given 3 days prior to LPS injections. LPS was stereotactically injected into the SNc on the third day, and 2-DG was administered for 7 consecutive days. On the 11th day, the mice were euthanized and brain tissue was collected for further study.

Rotarod test

Before the test, mice were trained on the rotarod for 10 min. During testing, the mice were placed on the rotarod with increasing speed from 4 rpm/min to 40 rpm/min, and the time when mice fell from the rotarod was recorded. The test for each mouse was recorded 3 times. The average of three trials was used as a statistical indicator [23].

Pole test

The pole used in this experiment is a wooden stick with a wooden ball on the top that has a rough surface. During testing, mice were placed on the wooden ball, and the time for the mice to climb from the ball to the base of the stick was recorded. The test for each mouse was recorded 3 times. The average of three trials was used as a statistical indicator.

Immunohistochemistry

The mice were euthanized, and normal saline was perfused into the left ventricle, followed by paraformaldehyde (4%) perfusion. After perfusion, the mouse brains were collected and fixed in phosphate-buffered paraformaldehyde for three days at 4 °C and then dehydrated in 30% sucrose solutions until they sank. The brain was frozen and cut into 20 µm slices with a freezing microtome. For immunofluorescence staining, the brain slices were washed in PBS and blocked with fetal bovine serum (FBS). Then, the samples were soaked with the appropriate antibody overnight. The next day, brain slices were washed in PBST for 30 min before or after incubation with secondary fluorescent antibodies for 2 hours in the dark. The samples were placed on a microscope slide and observed by confocal microscopy.

ADP/ATP ratio assay

An ADP/ATP Assay Kit (Sigma-Aldrich) was used in the ADP/ATP ratio assay. In brief, BV2 cells were seeded in a 12-well plate (1×10^5 cells/well). After LPS or/and compound stimulation for 16 hours, the cells were digested and collected in a centrifuge tube. Then, the cells were seeded in a 96-well plate with a white background (1×10^4 cells/well). The ATP reagent was prepared and added to each well. The samples were placed at room temperature for 1 min and measured by a luminescence reporter assay

system (Promega, Madison, WI, USA). The value was recorded as [(RLU)A]. After 10 min, the samples were measured again as [(RLU)B]. After recording [(RLU)B], 5 ml of ADP reagent was prepared and added to samples without delay. Then, the samples were incubated at room temperature for 1 min before the luminescence was detected as [(RLU)C]. The ADP/ATP ratio was calculated following the manufacturer's instructions.

NAD⁺/NADH assay

After LPS or/and compound stimulation for 16 hours, BV2 cells were digested and resuspended into two precooled tubes. Then, 100 µl of NAD extraction buffer or NADH extraction buffer was prepared and added to each tube. After heating at 60 °C for 5 min, 20 µl of assay buffer and 100 µl of the opposite extraction buffer were added to each sample, followed by centrifugation at 14,000 rpm for 5 min. Finally, 80 µl of working reagent was added to each sample, and the optical density was detected immediately (OD₀) and 15 min later (OD₁₅). NAD⁺/NADH was calculated according to the manufacturer's instructions.

Extracellular flux assays

Extracellular flux assays (Seahorse Bioscience, Chicopee, MA) were used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of BV2 cells. BV2 cells were plated in a Seahorse XF microplate at a density of 2.0×10^4 cells/well and cultured overnight. Then, the plate was balanced in a non-CO₂ incubator at 37 °C for 30 min. Meanwhile, Seahorse XF Glycolysis stress test kit assay medium was prepared, and glutamine was added to Seahorse XF Base Medium. Finally, the compounds were diluted in assay medium and added to the microplate. The ECAR and OCR were measured in an Agilent Seahorse XFe/XF96 or 24 Analyzer.

Coculture

BV2 cells were cultured in a 6-well plate at a density of 2.0×10^5 cells/well. Twenty-four hours later, the supernatant was collected as conditioned medium prior to LPS stimulation, and the compounds were stimulated for 6 hours. MES23.5 dopaminergic neural cells were cultured in a 96-well plate (2.0×10^4 cells/well), and after 12 hours, the conditioned medium was added to MES23.5 for 24 hours. Then, cell viability was measured by MTT assay and flow cytometry.

Flow cytometry

BV2 cells were treated with 2-DG for 30 min prior to lipopolysaccharide (LPS)-Alexa Fluor® 488 (L23351, Invitrogen, CA, USA) stimulation. After 2 hours, Cells were resuspended and washed in PBS for 3 times. The cell-associated fluorescence was measured by flow cytometry analysis (Beckman Coulter, Brea, CA, USA).

Statistical analysis

All data are presented as the mean $\pm$ standard deviation (S.D.) from at least three independent experiments and analyzed with GraphPad Prism software (version 8.0.1; San Diego, CA, USA). Comparisons between multiple groups were analyzed by one-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) with Tukey's test. $P < 0.05$ was considered statistically significant.

Results

LPS rapidly induces microglial aerobic glycolysis

The agonists of Toll-like receptors lead to a rapid increase in the glycolytic rate in dendritic cells [17]. We therefore determined whether LPS can also trigger aerobic glycolysis in microglial cells within a short period of stimulation. The results showed that the glycolytic rate (ECAR) was indeed upregulated within 150 min of LPS treatment (Fig. 1a). We then evaluated the expression of glycolytic genes, including Glut-1, HK2, and LDHA. Among these, the expression of Glut-1 and HK2 was upregulated, while the expression of LDHA was not affected in the LPS-activated BV-2 microglial cells (Fig. 1b-d). These results indicated that LPS can rapidly induce an increase in aerobic glycolysis in microglial cells.

Aerobic glycolysis is required for microglial inflammatory activation

To test whether glycolysis is required for microglial activation, we employed a small-molecule inhibitor or siRNA against glycolytic genes to interfere with glucose metabolism and measured proinflammatory gene expression as a functional output. Glycolytic inhibitors (2-DG and 3-BPA) significantly inhibited LPS-induced production of nitric oxide (NO) in a dose-dependent manner in BV-2 microglial cells as well as primary microglial cells in culture (Fig. 2a, c and s1a, c), while at the tested concentrations, these inhibitors did not affect microglial cell viability (Fig. 2b, d and s1b, d). The protein levels of proinflammatory genes, including TNF- α , IL-6, IL-1 β , iNOS and COX-2, were suppressed by pretreatment with glycolytic inhibitors in LPS-activated BV-2 microglial cells (Fig. 2e-g and s1e-g). Importantly, glycolytic inhibitors also inhibited proinflammatory genes at the transcriptional level in the LPS-activated BV-2 microglial cells (Fig. s2). Similar to glycolytic inhibitor treatment, knockdown of Glut-1, HK2 or LDHA significantly suppressed LPS-induced expression of TNF- α , IL-6, iNOS, COX-2 and IL-1 β at the transcriptional level in the BV-2 microglial cells (Fig. s3). These results suggest that inhibition of glycolysis reduces the expression of proinflammatory genes at both the protein and mRNA levels.

Glycolytic inhibitors suppress LPS-induced activation of NF- κ B signaling pathways

Transcriptional factor NF- κ B is a key regulator of the transcriptional expression of proinflammatory factors in LPS-activated microglial cells [24]. We therefore examined whether NF- κ B is involved in the inhibitory effects of glycolytic inhibitors on proinflammatory gene expression. The NF- κ B luciferase activity results showed that both 2-DG and 3-BPA dose-dependently suppressed LPS-induced NF- κ B transcriptional activation in BV-2 microglial cells (Fig. 3a and s4a). To further investigate the inhibitory mechanisms of glycolytic inhibitors on the NF- κ B signaling pathway, the upstream signaling pathways were evaluated. We first examined the effects of glycolytic inhibitors on the binding of Alexa488-

conjugated LPS to BV-2 microglial cells by flow cytometry analysis. The results showed that 2-DG did not affect FITC-LPS binding to the cell surface membranes of the BV-2 microglial cells (Fig. 3b). Next, we transfected various upstream molecules of the NF- κ B signaling pathway, including MYD88, RIP1, TRAF6, IKK β and p65, into HEK293T cells stably expressing the NF- κ B luciferase reporter construct and evaluated the effects of glycolytic inhibitors on the induced NF- κ B luciferase activity. As shown in Fig. 3c and s4b, overexpression of MYD88, RIP1, TRAF6, IKK β and p65 strongly induced NF- κ B luciferase activity in the HEK293T cells, and both 2-DG and 3-BPA significantly suppressed the induced NF- κ B luciferase activity. The kinase TAK1 not only phosphorylates IKK β , but also activates MAPKs, resulting in the activation of AP-1, which is another key transcriptional factor for the expression of proinflammatory genes in LPS-activated microglial cells[25]. We thus investigated the effects of glycolytic inhibitors on MAPK activation. As shown in Fig. 3d and s4c, neither 2-DG nor 3-BPA suppressed the phosphorylation of JNK, ERK and p38 induced by LPS. It was reported that TBK- $\text{IKK}\epsilon$ signaling plays crucial roles in TLR-derived early glycolytic reprogramming in dendritic cells. Therefore, we sought to determine whether glycolytic inhibitors suppress TBK1 phosphorylation in LPS-activated BV-2 microglial cells. The results showed that LPS-induced TBK1 phosphorylation was not affected by glycolytic inhibitors (Fig. 3e and s4d). Taken together, these results indicated that downstream of TAK1, IKK β and p65 may be targets of glycolytic inhibitors in LPS-driven NF- κ B activation.

Glycolytic inhibitors suppress LPS-induced IKK β activation by modulating AMPK/mTOR signaling in microglial cells.

Given that IKK β is known to be a major downstream signaling molecule in TLR4-mediated inflammatory responses, we next sought to determine whether glycolytic inhibitors can suppress LPS-induced IKK β activation. The results revealed that both 2-DG and 3-BPA inhibited LPS-induced phosphorylation of IKK α/β in BV-2 microglial cells in a dose-dependent manner (Fig. 4a and s5a). Since phosphorylation and degradation of I κ B α is mainly mediated by the activation of IKK β in LPS-activated microglial cells, we explored the effect of glycolytic inhibitors on phosphorylation and degradation of I κ B α . As shown in Fig. 4a and s5a, both 3-BPA and 2-DG reduced the phosphorylation and degradation of I κ B α in a dose dependent manner. We also observed that knocking down HK2 inhibited LPS-induced phosphorylation and the subsequent degradation of I κ B α in BV-2 microglial cells (Fig. 4b). These results indicated that the anti-inflammatory effects of glycolytic inhibitors may be related to IKK β inhibition.

Since only one of two downstream molecules of TAK1 was inhibited by the glycolytic inhibitors, we speculated that another upstream regulator of IKK β may be involved in the anti-inflammatory activity of glycolytic inhibitors. AMPK/mTOR has been reported to positively regulate IKK β activation [26, 27]. Therefore, we investigated the effects of glycolytic inhibitors on AMPK phosphorylation and mTOR phosphorylation in LPS-activated BV-2 microglial cells. Both 2-DG and 3-BPA inhibited the phosphorylation of mTOR and increased the phosphorylation of AMPK in the LPS-activated BV2 microglial cells (Fig. 4c and s5b). Since AMPK is directly regulated by the ADP/ATP ratio, we also measured the ADP/ATP ratio in the BV2 cells. 2-DG and 3-BPA increased the ADP/ATP ratio both in the activated and inactivated BV2 microglial cells, corresponding to AMPK phosphorylation (Fig. 4d and s5c).

These results revealed that glycolytic inhibitors suppressed IKK phosphorylation by increasing AMPK phosphorylation and inhibiting mTOR phosphorylation.

Glycolytic inhibitors suppress LPS-induced transcriptional activity of NF- κ B via inhibition of NAD⁺/SIRT1 mediated p65 acetylation

Since glycolytic inhibitors reduced p65 overexpression-induced NF- κ B transcriptional activation, we tested the effects of glycolytic inhibitors on p65 nuclear translocation. The results of the nucleus-cytoplasm fraction assay revealed that both 2-DG and 3-BPA markedly inhibited LPS-induced nuclear distribution of the p65 subunit of NF- κ B (Fig. 5a). The acetylation of p65 at lysine 310 was demonstrated to promote the transcriptional activity of NF- κ B [28]. Therefore, we asked whether a glycolytic inhibitor can decrease p65 acetylation levels at lysine 310 in LPS-activated BV-2 microglial cells. The results showed that LPS increased the acetylation of p65 levels and that 2-DG significantly decreased LPS-induced p65 acetylation (Fig. 5b). This finding was confirmed in the TNF- α treated HEK 293T cells that were cotransfected with p300 and the p65 subunit of NF- κ B (Fig. 5c). p65 acetylation is suppressed by Sirtuin 1 (SIRT1), whose activity is dependent on intracellular NAD⁺ levels, which are regulated by glucose metabolism [29, 30]. We therefore tested whether the inhibitory effects of 2-DG on NF- κ B activation were dependent on SIRT1 activation. As shown in Fig. 5d, 2-DG rescued the LPS-induced decrease in the NAD⁺/NADH ratio in BV-2 microglial cells. Knockdown of SIRT1 partly blocked the inhibitory effect of 2-DG on inflammatory cytokine production (IL-1 β and TNF- α) in LPS-activated BV-2 microglial cells (Fig. 5e-g). These results indicate that the reduction of NAD⁺-mediated p65 acetylation is involved in the anti-inflammatory activity of inhibition of glycolysis.

Neuroprotective effects of glycolytic inhibitors in activated microglial CM /neuron coculture model

Several lines of evidence have indicated that proinflammatory factors derived from overactivated microglial cells can induce injury to or the death of surrounding dopaminergic neuronal cell *in vitro* and *in vivo* [2]. Since glycolytic inhibitors exhibited anti-inflammatory activity, we speculated that the inhibition of glycolysis might mitigate microglial activation-induced dopaminergic neuronal cell death. To examine this possibility, a BV-2 microglial cell-conditioned media/MES23.5 dopaminergic neuronal cell coculture system was employed [31]. As shown in Fig. 6a and s6a, while MES23.5 cells were incubated with CM from LPS-stimulated BV-2 microglial cells, the cell viability was significantly decreased, and pretreatment with glycolytic inhibitors obviously attenuated this effect. It has been reported that 2-DG attenuated rotenone- or Fe²⁺-induced SK-N-MC neuroblastoma cell death [20]. We thus examined whether glycolysis directly ameliorated the MPP⁺-induced reduction in MES23.5 cell viability. The results showed that neither 2-DG or 3-BPA at the tested concentrations blocked MPP⁺-induced MES23.5 cell death (Fig. 6b and s6b). These results indicated that the inhibition of glycolysis is neuroprotective, which is likely attributable to the suppression of microglial inflammatory activation.

Glycolytic inhibitor 2-DG ameliorates neuroinflammation and inflammation-mediated dopaminergic cell loss in an LPS-induced mouse PD model

Given the anti-inflammatory and subsequent neuroprotective effects of the glycolytic inhibitors *in vitro*, we further assessed the inhibitory effects on neuroinflammation in an intracranial LPS-induced mouse PD model (Fig. 7a). Mice were administered a peritoneal injection of 2-DG (400 mg/kg daily for three consecutive days) before and after stereotactic injection of LPS (5 mg/kg). After 7 days of post-LPS injection, microglial activation and DA neuron loss were assessed by coimmunostaining of Iba1 (a microglial cell marker) and TH (a dopaminergic neuron marker). The results showed that LPS administration elicited an obvious increase in Iba1-positive cells, which was accompanied by significant TH-positive cell loss in the substantia nigra region. LPS-induced microglial activation and subsequent dopaminergic cell loss were significantly attenuated by 2-DG treatment (Fig. 7b-d). These results indicated that the inhibition of glycolysis ameliorated neuroinflammation and subsequent dopaminergic cell death.

Glycolytic inhibitor 2-DG significantly ameliorates MPTP-induced behavioral deficits in mice

It is well known that damaged neurons may elicit surrounding microglial activation. In turn, activated microglia facilitate neuronal damage by releasing a variety of neurotoxic factors [2]. Therefore, inhibition of microglial cell activation may block a self-amplifying cycle of neuronal injury and microglial activation, which eventually endows neuroprotection [3]. Thus, we investigated whether 2-DG improved MPTP-induced substantia nigra neuronal cell damage and subsequent microglial activation (Fig. 8a). As expected, pretreatment with 2-DG significantly mitigated MPTP-induced mouse behavioral defects (Fig. 8b, c). In addition, immunofluorescence and Western blot analysis showed that 2-DG treatment markedly reduced TH-positive cell loss and microglial cell activation (Fig. 8d-g). These results indicated that inhibition of glycolysis also blocked the vicious cycle of dopaminergic cell damage and neuroinflammation *in vivo*.

Discussion

In the present study, it was found that inhibition of aerobic glycolysis suppressed LPS-induced microglial proinflammatory gene expression at both the mRNA and protein levels via the inhibition of NF- κ B transcriptional activity. The mechanistic studies indicated that the activation of AMPK and subsequent inhibition of mTOR/IKK β signaling and SIRT1-mediated p65 deacetylation may be involved in the inhibitory activity of glycolytic inhibitors on LPS-induced NF- κ B transcriptional activation. Furthermore, in both LPS- and MPTP-induced PD models, 2-DG significantly attenuated microglial activation and dopaminergic cell loss.

The metabolic shift from oxidative phosphorylation to aerobic glycolysis has emerged as a hallmark of the proinflammatory activation of microglial cells. However, it is largely unknown whether this reprogramming is required for the expression of proinflammatory genes in LPS-activated microglial cells. We found that the knockdown of glycolytic enzymes and application of small-molecule inhibitors both blocked LPS-induced microglial activation, suggesting that glycolysis is essential for microglial inflammatory activation. Previously, it was reported that aerobic glycolysis controlled IFN- γ

production upon the binding of GAPDH to the AU-rich elements of the 3'-untranslated region of IFN- γ mRNA in T cells [32]. It was also reported that GAPDH-ARE binding is critical for posttranslational control of TNF- α production in LPS-primed monocytes [33]. However, in dendritic cells, 2-DG suppressed proinflammatory cytokines, including TNF- α , IL-6 and IL-12, at posttranscriptional levels, through GAPDH-ARE binding independent mechanisms [17]. Unexpectedly, in our results, we found that glycolytic inhibition reduced the expression of proinflammatory genes at both the mRNA and protein levels, suggesting that transcriptional mechanisms are also involved in the regulation of microglial activation by glycolysis.

During microglial activation, the expression of proinflammatory genes is mainly controlled by NF- κ B and AP-1, which are key transcriptional factors that regulate the expression of a number of genes critical for inflammatory responses. After binding with LPS, TLR4 elicits TAK1 activation by recruiting adaptor molecules and protein kinases such as MyD88, IRAKs and TRAF6, resulting in phosphorylation of IKK β and MAPKs. The activation of MAPKs, including JNK, p38 and ERK1/2, elicits transcriptional factor AP-1 activation, ultimately leading to the expression of proinflammatory genes. In the present study, we found that glycolytic inhibitors did not reduce LPS-induced MAPK phosphorylation but suppressed IKK β phosphorylation, suggesting that TAK1 is not involved in the anti-inflammatory activity of glycolytic inhibitors in microglial cells. In addition, we also found that glycolytic inhibitors blocked both IKK β and p65 overexpression but not TAK1 overexpression-induced NF- κ B luciferase activity and inhibited LPS-induced p65 nuclear translocation, the phosphorylation and degradation of I κ B α and the phosphorylation of p65. These results strongly suggest that IKK β and p65 may be targets of glycolytic inhibition.

Given that TAK1 was not involved in the anti-inflammatory effects of the glycolytic inhibitors in microglial cells, another signaling molecule may be upstream of IKK β and p65. There are several lines of evidence showing that mTOR, a downstream molecule of AMPK, is involved in the activation of NF- κ B signaling pathways [34–36]. mTOR promotes NF- κ B transcriptional activity by directly interacting with IKK, and the inhibition of mTOR by rapamycin reduces proinflammatory gene expression by blocking NF- κ B activation in microglial cells [37]. Our previous study also demonstrated that rapamycin significantly suppressed IKK β phosphorylation in LPS-activated BV-2 microglial cells [27]. In the present study, we found that inhibition of glycolysis suppressed LPS-induced mTOR phosphorylation. Indeed, 2-DG has been shown to inhibit mTOR in some cancer cell lines [38]. mTOR phosphorylation is mainly regulated by the upstream molecule AMPK [37]. AMPK is a highly conserved protein kinase complex that is a key component of cellular energy metabolism in eukaryotes [37]. AMPK induces rapid inhibition of mTOR signaling by directly phosphorylating TSC2 and raptor. There is growing evidence showing that activation of AMPK significantly inhibits microglial inflammatory activation by blocking the NF- κ B signaling pathway [39, 40]. AMPK is activated by the phosphorylation of Thr172 at a high ADP/ATP ratio [39]. In the present study, glycolytic inhibitors activated AMPK by increasing the ADP/ATP ratio, which was combined with the inhibition of mTOR phosphorylation and IKK β in microglial cells, suggesting that the AMPK/mTOR/IKK β signaling pathway is at least partly involved in the anti-inflammatory mechanisms of glycolytic inhibition. In addition to phosphorylation, the acetylation of p65 is also crucial in regulating NF- κ B transcriptional activation under inflammatory conditions [41]. The acetylation of p65 is mainly mediated by different

histone acetyltransferases, including CBP and p300 [41]. The acetylation status of p65 is also negatively controlled by histone deacetylases, including SIRT1 [28]. SIRT1, an NAD⁺-dependent protein deacetylase, can deacetylate p65 at lysine 310, thereby inhibiting NF-κB-mediated macrophage activation [28]. SIRT1 activator resveratrol or overexpression of SIRT1 can significantly inhibit microglial inflammatory activation by suppressing NF-κB transcriptional activity [42]. In the present study, 2-DG blocked the LPS-induced decrease in the NAD⁺/NADH ratio and p65 acetylation, resulting in the inhibition of LPS-induced NF-κB activation. These results suggest that inhibition of the NAD⁺-dependent SIRT1/NF-κB pathway might contribute to the anti-inflammatory effects of glycolytic inhibition.

Although neuronal cells are critical to the progression of neurodegenerative diseases and are the main targets of these diseases, microglial cell-mediated inflammatory microenvironments are actively involved in the pathogenesis and progression of neurodegenerative diseases, including PD [43]. Overactivated microglial cells create a neuroinflammatory microenvironment by releasing various proinflammatory factors, thereby ultimately contributing to neurodegenerative progression [44]. Many studies have demonstrated that inhibition of microglial activation by anti-inflammatory compounds or modulation of the expression of some genes attenuated neuronal cell injuries under neurodegenerative conditions, suggesting that suppression of microglial activation may be considered a potential therapeutic approach to neurodegenerative diseases [45–47]. In the present study, we found that the glycolytic inhibitor 2-DG significantly inhibited neuronal cell death induced by conditioned media from activated microglial cells. We also found that pretreatment with 2-DG significantly attenuated dopaminergic cell death and microglial cell activation. Consistent with our study, a previous report demonstrated that 2-DG reduced MPTP-induced TH-positive cell death by increasing the expression of the stress-responsive proteins GRP78 and HSP70 in neuronal cells [20]. However, we did not find a protective effect of 2-DG on MPP⁺-induced neuronal death. On the other hand, many studies have suggested that 2-DG may inhibit cell growth or promote cell apoptosis in various tumor cells [48], suggesting that the effect of 2-DG on cell survival is dependent on the cell type or nature of the stimuli. It is suggested that stereotactic injection of LPS is able to induce loss of DA neurons. We found that 2-DG significantly ameliorated LPS-induced DA loss *in vivo*. Thus, it is reasonable to conclude that 2-DG ameliorates DA neuron loss *in vivo* by inhibiting microglia-mediated neuroinflammation.

Conclusions

In summary, the present study demonstrated that 1) inhibition of glycolysis suppressed the expression of proinflammatory genes in LPS-activated microglial cells *in vitro* and *in vivo*; 2) inhibition of NAD⁺/SIRT1/p65 acetylation involved the anti-neuroinflammatory activity of the glycolytic inhibitors; 3) AMPK/mTOR/IKK signaling involved the anti-neuroinflammatory response of the glycolytic inhibitors; and 4) glycolytic inhibitor 2-DG exhibited neuroprotective effects in LPS or MPTP mouse PD models. Therefore, inhibition of glycolysis may be a potential therapeutic target for the treatment of neuroinflammation-related diseases.

Abbreviations

LPS: lipopolysaccharide; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 2-DG: 2-dexoy-D-glucose; 3-BPA: 3-bromopyruvic acid; NF- κ B: Nuclear factor kappa B; HK β : Hexokinase 2; Glut-1: Glucose transporter 1; mTOR: Mammalian target of rapamycin; SIRT1: Sirtuin 1; PD: Parkinson's disease; IFN-g: Interferon-g; TNF- α : Tumor necrosis factor- α ; NO: Nitric oxide; (PG)E₂: prostaglandin; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; ROS: Reactive oxygen species; ECAR: Extracellular acidification rate; LDHA: lactate dehydrogenase; iNOS: Inducible nitric oxidesynthase; COX-2: Cyclooxygenase 2; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; CM: conditional medium.

Declarations

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by approved by the Institutional Animal Care and Use Committee of Soochow University.

Consent for publication

Not applicable

Conflict of interests

The authors declare no conflict of interests.

Author contributions

X.Z. and L-T. Z designed experiments; J. C., R. Z., Y. K., Z. X., and R. S. performed experiments; Y.H., J. C. and R. Z. analyzed data; X. Z. and L-T. Z discussed data and wrote the manuscript.

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Figures

Figure 1

LPS rapidly induced microglial aerobic glycolysis. a Real-time changes in the ECAR of the BV-2 microglial cells treated with LPS (0.1 µg/ml) (arrow indicates initiation time of LPS treatment). BV-2 microglial cells were treated with LPS for the indicated times, and then, the expression of Glut-1 (b), HK-2 (c) and LDHA (d) was measured by quantitative real-time PCR (Q-PCR). The values are the means ± S.D. of 3 different experiments (n=3). *p<0.05, **p<0.01, compared to the control group.

Figure 2

Aerobic glycolysis is required for microglial inflammatory activation. BV2 microglial cells or primary microglia cells were pretreated with 2-DG (600-1000 μM) for 30 min, followed by LPS (200 ng/mL) treatment for 24 hours. The nitrite in the cell culture medium was quantified using the Griess reaction (a, BV-2 cells; c, Rat primary microglia). Cell viability was determined by MTT assay (b, BV-2 cells; d, rat primary microglia). The concentrations of IL-6 (e) and TNF- α (f) were analyzed by ELISAs. The expression of iNOS, COX-2 and IL-1 β (g) was determined by Western blotting (upper) and the relative protein levels were quantified by densitometry analysis (lower). The values are the means $\pm$ S.D. of 3 different experiments (n=3). * $p < 0.05$, ** $p < 0.01$, compared to the NONE group or LPS alone group.

Figure 3

Glycolytic inhibitors suppress the inflammatory response by negatively regulating NF-κB signaling. BV-2 cells stably expressing NF-κB luciferase reporter construct were pretreated with 2-DG (600-1000 μM) for 30 min, followed by LPS (200 ng/mL) stimulation for 16 hours. The transcriptional activity of NF-κB was determined by luciferase reporter assay (a). BV2 cells were pretreated with 2-DG for 30 min, followed by lipopolysaccharide (LPS)-Alexa Fluor® 488 treatment for 2 hours. The binding efficiency was quantified using flow cytometry (b). c Luciferase activity was detected in HEK293T cells transfected with an NF-κB

luciferase reporter and the indicated plasmids. d BV-2 cells were pretreated with 2-DG (1000 μ M) for 30 min, followed by LPS (200 ng/mL) treatment for 30 min. The expression of p-JNK, JNK, p-ERK, ERK, p-p38, and p38 was detected by Western blotting (left) and the relative protein levels were quantified by densitometric analysis (right). e BV-2 cells were treated with 2-DG (1000 μ M) for 30 min prior to LPS stimulation for 30 min. The expression of p-TBK1 was analyzed by Western blotting (left) and the relative protein levels were quantified by densitometric analysis (right). The values are the means $\pm$ S.D. of 3 different experiments (n=3). * $p < 0.05$, ** $p < 0.01$ compared to the LPS alone group or control group.

Figure 4

Glycolytic inhibitors suppressed LPS-induced IKK β activation by modulating AMPK/mTOR signaling in microglial cells. a BV-2 cells were pretreated with 2-DG (600-1000 μ M) for 30 min, followed by LPS (200

ng/mL) treatment for 15 min. The expression of p-IKK α / β , p-I κ B- α and I κ B- α was analyzed by Western blotting (upper) and the relative protein levels were quantified by densitometric analysis (lower). b BV2 cells were treated with control siRNA or si-HK2. After 48 hours, the cells were stimulated with LPS (200 ng/ml) for the indicated times (0, 5, 10, 20, 30, 60 min), and the expression of HK2, p-I κ B- α and I κ B- α was determined by Western blotting (upper) and the relative protein levels were quantified by densitometric analysis (lower). c BV-2 cells were pretreated with 2-DG (600-1000 μ M) for 30 min, followed by LPS (200 ng/mL) treatment for the indicated times. After 1 hour, the expression of p-AMPK and p-mTOR (total mTOR) was determined by Western blotting (upper) and the relative protein levels were quantified by densitometric analysis (lower). d After 16 hours, the ADP/ATP ratio was calculated as described in the methods and materials section. The values are the means $\pm$ S.D. of 3 different experiments (n=3). * p < 0.05, ** p < 0.01, compared to the LPS group.

Figure 5

Glycolytic inhibitors suppressed LPS-induced transcriptional activity of NF- κ B via inhibition of NAD⁺/SIRT1-mediated p65 acetylation. a BV-2 cells were pretreated with 2-DG (1000 μ M) or 3-BPA (100 μ M) for 30 min, followed by LPS (200 ng/mL) treatment for 1 hour. The expression of NF- κ B p65 and p-NF- κ B p65 in the cytoplasm and nucleus was analyzed by Western blotting (left) and the relative protein levels were quantified by densitometric analysis (right). b BV2 cells were pretreated with 2-DG (1000 μ M)

for 30 min followed by LPS (200 ng/mL) treatment for 1 hour. The extracts were subjected to immunoprecipitation (IP) with anti-p65 antibody or anti-IgG followed by immunoblotting analysis with anti-acetylated-p65 antibody and anti-p65 antibody. The relative expression of ac-p65 was quantified by western blotting (upper) and the relative protein levels were quantified by densitometric analysis (lower). c HEK293T cells were cotransfected with p300 and p65 plasmids for 24 hours, followed by 2-DG (1000 μ M) treatment for 30 min and then treated with TNF- α (10 ng/ml) for 1 hour. The extracts were subjected to immunoprecipitation (IP) with anti-p65 or anti-IgG followed by immunoblotting analysis with anti-acetylated-p65 antibody and anti-p65 antibody. The expression of ac-p65 was determined by western blotting (upper) and the relative protein levels were quantified by densitometric analysis (lower). d BV-2 cells were pretreated with 2-DG (600-1000 μ M) for 30 min, followed by LPS (200 ng/mL) treatment for 16 hours. The NAD⁺/NADH ratio was measured as described in the methods and materials section. e-g BV-2 cells were transduced with si-SIRT1 (40 nM) or control siRNA (siCtrl) for 48 hours, followed by LPS (200 ng/mL) treatment for 6 hours. The mRNA expression of SIRT1, TNF- α and IL-1 β was measured by qPCR. The values are the means $\pm$ S.D. of 3 different experiments (n=3). * p<0.05, ** p<0.01 compared to the LPS, siCtrl or siCtrl + LPS group respectively.

Figure 6

Glycolytic inhibitors reduce CM of activated microglial cell-induced MES23.5 neuroblastoma cell death. a BV-2 cells were pretreated with 2-DG (600-1000 μ M) for 30 min, followed by LPS (200 ng/mL) treatment for 6 hours. The culture media was removed, and fresh medium was added. After 24 hours of incubation, culture medium was added to the MES23.5 cells. Cell viability was determined by MTT assay. b MES23.5 cells were treated with 2-DG (62.5-500 μ M) and with MPP+ (1000 μ M) for 24 hours. Cell viability was

determined by MTT assay. The values are means $\pm$ S.D. of 3 different experiments (n=3). * p <0.05, ** p <0.01, compared to the LPS or MPP+-alone group.

Figure 7

Glycolytic inhibitor 2-DG ameliorates neuroinflammation and inflammation-mediated dopaminergic cell loss in an LPS-induced mouse PD model. a Schematic diagram of drug administration. Mice were sequential intraperitoneally injected with 2-DG (400 mg/kg) or saline for 10 days. On the third day, mice

were stereotactically injected with LPS (5 mg/kg) or saline (NS). After 10 days, the mice were euthanized for IHC analysis. b Immunostaining for TH and Iba1 in the SNc, n = 3 mice per group (scale bar, 80 μ m). c-d Quantitative analysis of TH-positive and Iba-1-positive cells in the SN. The data are presented as the means $\pm$ S.D. * p <0.05, ** p <0.01, compared as indicated above.

Figure 8

Glycolytic inhibitor 2-DG significantly ameliorates MPTP-induced behavioral deficits in mice. a-c Mice were sequentially intraperitoneally injected with 2-DG (400 mg/kg) for 3 days and then sequentially intraperitoneally injected with MPTP (30 mg/kg), 2-DG (400 mg/kg) or saline (NS) for 7 days. Behavioral tests after MPTP-induced bradykinesia were conducted on the 11th day. Mice were euthanized and the brains were collected on the 13th day; n = 10 mice per group. d Immunostaining for TH and Iba-1 in the SN; n=5 mice per group. Scale bar: 100 μ m. e-f Quantitative analysis of the TH-positive (e) and Iba-1-positive (f) cells in the SN. g TH protein expression in the striatum (STR) and SN was determined by Western blotting (left) and the relative protein levels were quantified by densitometric analysis (right); n=3 mice per group. The data are presented as the means $\pm$ S.D. * p <0.05, ** p <0.01, compared to the MPTP group.

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