Inhibition of Voltage-Gated Hv1 Alleviates LPS-Induced Neuroinflammation via Regulation of Microglial Metabolic Reprogramming

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Research

Keywords: neuroinflammation, metabolic reprogramming, cognitive deficits, microglia, hippocampus

Posted Date: January 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1175878/v1

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Abstract

Background

Neuroinflammation plays an important role in the onset and advancement of cognitive loss and neurodegenerative disorders. The voltage-gated H channel (Hv1) has been reported to be involved in microglial activation and act as key drivers of neuroinflammation. This study aims at evaluating the mechanism of Hv1 involvement in neuroinflammation and the therapeutic potential of Hv1 inhibitor, 2-guanidinobenzimidazole (2-GBI), in a model of lipopolysaccharide (LPS)-induced neuroinflammation.

Methods

We investigated the influence of Hv1 inhibitor (2-GBI) on the generation of reactive oxidative species (ROS), metabolic reprogramming, and inflammatory mediators in vitro and examined the therapeutic potential of 2-GBI on microglial activation and hippocampal neuroinflammation in vivo. Novel object recognition and Y-maze were employed to assess cognitive function.

Results

2-GBI reduced the LPS-induced proinflammatory response and aerobic glycolysis in microglia. HIF1α overexpression mediated aerobic glycolysis reprogramming alleviated by 2-GBI. We reported that Hv1 inhibitor exerted a protective effect on LPS-induced neuroinflammation through the ROS/HIF1α and PI3K/AKT/HIF1α pathways -mediated aerobic glycolysis. The cell death of PC12 induced by microglia-mediated neuroinflammation was reversed in a transwell co-culture system by 2-GBI. Furthermore, in vivo results suggested that 2-GBI mitigated the neuroinflammatory processes and recognition injury through regulation of microglial metabolic reprogramming.

Conclusion

2-GBI protects LPS-induced neuroinflammation, neuronal cell death, and subsequently reverses the hippocampus-dependent cognitive deficits through regulation of microglial metabolic reprogramming. Taken together, these results demonstrate a key role for Hv1 in driving a pro-inflammatory microglia phenotype in neuroinflammation.

Background

Neuroinflammation is considered to be a key element to almost all neurodegenerative disorders [1]. Myeloid-derived microglia and macrophages reside within the brain, which play an indispensable role in immune responses and homeostasis maintenance in the central nervous system, as well as mediates during a neuroinflammatory process [2]. In response to immunological challenges, microglia readily
becomes activated as characterized by marked morphological dynamics, and modulation of neuronal activity that impacts inflammation-mediated neuronal degeneration [3, 4]. Activated microglia can secrete various cytotoxic factors and pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) [5]. Nevertheless, exacerbated microglial activation may additionally stimulate the inflammatory responses of glial cells and lead to potentially long-lasting detrimental effects on the brain [6]. Therefore, attenuating microglia-mediated inflammatory response and neuronal injury has become an important target for improving the process of neurodegeneration.

Microglia usually change their phenotype and metabolic state in response to immune challenge [7, 8]. During the classic inflammatory activation process, microglia cellular metabolism is reprogrammed from oxidative phosphorylation (OXPHOS) toward glycolysis even in the presence of oxygen, a phenomenon also known as the Warburg effect. Whereas a high basal mitochondrial oxygen consumption rate (OCR) is possessed by alternatively activated macrophages [9, 10]. Several reports have demonstrated that pharmacologic inhibition of glycolysis could blunt the M1 polarization in macrophages [11, 12].

The voltage-gated proton channel Hv1 (also termed VSOP), encoded by Hvcn1, is mainly expressed in immune cells such as neutrophils, macrophages, B lymphocytes, and microglia [13]. Hv1 plays an essential role in alleviating the coincident intracellular acidosis via NADPH (NOX2)-dependent extrusion of hydrogen (H+) protons [14]. Hv1 is electively expressed in microglial cells in the central nervous system [15]. The Microglial Hv1 proton canal has been suggested as an essential target that incorporates inflammation into the injured microenvironment during ischemic stroke, traumatic brain injury and spinal cord injury [16–18]. Hv1-deficient mice exhibited reduced inflammation and long-term neuroprotection in preclinical experimental models [19]. However, the molecular mechanisms responsible for Hv1-mediated microglial activation and neuroinflammation have not been fully elucidated. In this study, we demonstrated a novel evidence that Hv1 acts as the upstream regulator to promote metabolic reprogramming and subsequent neuroinflammation through HIF-1α. Inhibition of Hv1 by a specific Hv1 inhibitor, 2-guanidinobenzimidazole (2-GBI), not only suppressed lipopolysaccharide (LPS)-induced microglial activation and neuroinflammation \textit{in vitro} and \textit{in vivo}, but also reduced cell apoptosis, and alleviated memory impairment in LPS-injected mice.

\section*{Methods}

\subsection*{Chemicals}

LPS from \textit{E. coli} \textit{O111:B4} (L3012), Cobalt(II) chloride hexahydrate (CoCl$_2$ · 6H$_2$O) and 2-GBI (G11802) used for all experiments were obtained from Sigma-Aldrich (St. Louis, MO) and upon dissolution in DMSO (Sigma-Aldrich) produced 1 mg/mL stock solutions.

\subsection*{BV2 Cell culture}

Murine BV2 microglia and rat PC12 cells were procured from the ATCC and were subjected to culturing in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with fetal bovine
serum (FBS, 10%). Culturing of cells was carried out in a 5% CO$_2$ incubator at 37°C. Before the experiment, seeding of the cells was carried out into 12- or 96-well plates and incubated for 12 hrs. BV2 cells were pre-treated with 200 µM 2-GBI, a selective and state-dependent blocker of Hv1 channels for 1 hr, and then treated with LPS 50 ng/mL for 4 and 6 hrs. At various times following LPS exposure, cells were harvested and used in a metabolic assay or immunoblot analysis of neuroinflammation and glycolysis. For CoCl$_2$ treatment, BV2 cells were pre-treated with 200 mM CoCl$_2$ for 24 hrs, then treated with 200 µM 2-GBI for 9 hrs.

**Preparation of primary murine microglia**

C57BL/6 mice of 24 hrs of age were used to establish primary microglial cells according to the previously described method [20]. Microglial cells were suspended and obtained on day 11-13, by shaking the flasks for 10 min at 180 rpm and 37°C. The mature microglial cells were seeded within plates at a density of $2 \times 10^5$/cm$^2$ and before 2-GBI (200 µM) were used for pre-treating the primary microglia 1 hr, followed by treatment with 50 ng/mL LPS in DMEM supplemented with 10% FBS for 4 hrs and 6 hrs. Following LPS exposure, cells were harvested at various times and used in metabolic assay or immunoblot analysis of neuroinflammation and glycolysis.

**NO assay**

The level of accumulated nitrite (NO$_2^-$), a metabolite of NO is measured using the Griess assay, using the Griess reagent in the culture supernatant. BV2 cells and primary microglia were incubated overnight after being seeded in a 96-well plate at a density of $1 \times 10^5$ cells in each well. The cell culture supernatant was gathered followed by measurement of the concentration of NO using the Griess reagent.

**Western Blotting**

Western blot was conducted as elaborated in detail earlier [21]. 4-20% Sure PAGE Bis-Tris gels (GenScript, Nanjing, China) were employed to run the protein sample (40 µg) which are later placed onto PVDF membranes (88585, Thermo Scientific, USA), blocked overnight in 0.1% Tween-20 in TBS (pH 7.4), 5% milk at 4°C, followed by incubation with primary antibodies: Caspase-3 (14220, Cell Signaling Technology, 1:1000), AKT (4691, Cell Signaling Technology, 1:1000), phospho-AKT (4060, Cell Signaling Technology, 1:1000), p44/42 MAPK (ERK1/2) (4695, Cell Signaling Technology, 1:1000), phospho-ERK1/2 (4370, Cell Signaling Technology, 1:1000), PI3K (4249, Cell Signaling Technology, 1:1000), phospho-PI3K (17366, Cell Signaling Technology, 1:1000), phospho-mTOR-S2448 (AP0115, ABclonal, 1:1000), iNOS (A18247, ABclonal, 1:1000), COX2 (12282, Cell Signaling Technology, 1:1000), IL-1β (Ab9277, Abcam, 1:1000), TNFα (ab9277, Abcam, 1:1000), hexokinase 2 (HK2) (A0994, ABclonal, 1:1000), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) (ab181861, Abcam, 1:1000), PSD-95 (ab18285, Abcam, 1:1000), synaptophysin (ab32127, Abcam, 1:1000), hypoxia-inducible factor 1α (HIF1α) (A16873, ABclonal, 1:1000), and β-actin (AC026, ABclonal, 1:3000) in 4°C overnight to probe targeted proteins. Horseradish peroxidase-conjugated secondary antibodies (AS014, ABclonal, 1:4000) were used to combine primary antibodies and the reaction was detected with a BeyoECL Plus ECL Kit.
(Beyotime, China). The immunoblots were then scanned and quantified by the ImageJ software. The band intensity values of the target proteins were normalised to that of β-actin.

**Metabolic assays**

Within the culture medium, the lactate levels were ascertained by employing a Lactate Assay Kit (BioAssay Systems) following the manufacturer's instructions. For analyzing the extracellular acidification rates (ECAR) (103020-100, Seahorse Biosciences/Agilent Technologies, Billerica, MA, USA) and OCR (103015-100, Seahorse Biosciences/Agilent Technologies, Billerica, MA, USA), an XF96 extracellular flux analyzer (Seahorse Biosciences/Agilent Technologies, Billerica, MA, USA) was used to analyze primary microglia and BV2 cells. Individual wells containing 12,000 cells were then cultured for 12 hrs at 37°C in a 5% carbon dioxide condition in an incubator. Following LPS exposure, cells were used for metabolic assays.

**Reactive oxygen species (ROS) measurement**

A 12-well plate was used to culture cells. 300µL of FBS-free DMEM medium supplemented with DCFH-DA was added. The 12-well plate was allowed to sit in an incubator within a 5% carbon dioxide atmosphere at 37°C for 20 minutes. After irradiation, flow cytometry (BD Accuri™ C6 Plus, BD Biosciences) was used to analyze the cells and the mean fluorescence was estimated using FlowJo software (FlowJo, LLC, Ashland OR, USA).

**Co-cultures of PC12 neurons and BV2 microglia**

As previously described [22], the transwell co-culture system was conducted by a semi-permeable 0.4-µm membrane (Costar, NY, USA). First, Cells were pre-treated with 200 µM 2-GBI for 1 hr and subsequently incubated with LPS (50 ng/mL) for the 6 h. After treatment with LPS and/or 2-GBI, BV2 microglia were seeded in the upper chamber at a density of 1×10^5 cells in 1 mL serum-free medium, and PC12 cells were plated in the lower chamber at a density of 2×10^5 cells in 2 mL serum-free medium. After co-cultivation for 24 hrs, PC12 cells were harvested for further analysis.

**Cell viability assay**

PC12 cell viability in response to the supernatants of BV2 cells was determined by CCK-8 assay according to the manufacturer's instructions (Dojindo, Tokyo, Japan). Briefly, PC12 cells were cultivated at a density of 10,000 cells/well in 96-well plates. 10 µL of CCK-8 solution was added to each well of the plate, and the plates were incubated for 0.5 h. Assay plates were shaken on an orbital shaker for 2 min. Data acquisition was then performed using a microplate reader.

**Detection of PC12 apoptosis by TUNEL assay**

After co-cultivation for 24 hrs, PC12 cells were harvested for TUNEL analysis. Apoptosis was determined by the DeadEnd terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL, Beyotime, China) according to manufacturer’s instructions. Apoptotic nuclei were normalized to total DAPI (Beyotime, China)-stained nuclei.
Animals experiments

The Institutional Animal Care and Use Committee of Peking University (Permit No: 2011-0039) duly passed all animal experiments. C57BL/6J male mice, 7 weeks of age, were bought from the Medical Laboratory Animal Centre of Guangdong Province (approval No. SCXK (Yue) 2008–0002), and acclimatized under standard condition (12/12-h light-dark cycle, 55 ± 5% humidity, 23 ± 1°C) for one week with unrestricted access to food and water.

Four groups of the eight-week-old male mice were made (12-14 mice in each group): namely LPS (5 mg/kg, intraperitoneally), saline-treated, 2-GBI (1 mg/kg, intraperitoneally), and LPS + 2-GBI. Following 6 h of injecting LPS and 2-GBI, brain tissues were gathered and stored until further investigation at freezing temperatures (− 80°C). Following 24 h of injecting LPS and 2-GBI, behaviors analysis of the mice was carried out (described in detail below), following which they were sacrificed.

Immunofluorescence

As elaborated earlier [23], immunofluorescence analysis was carried out. Dissection of the brains was done, and the tissue processing was carried out as explained earlier [24]. Overnight incubation of the sections with the mentioned primary rabbit anti-PFKFB3 antibody (1:200, ab181861, Abcam) and Iba1 (1:200, 016-26721, Wako) were carried out at 4°C. Secondary antibodies conjugated to Alexa Fluor 594-labeled goat anti-mouse IgG (1:500, ab150116, Abcam) and Alexa Fluor 488-labeled goat anti-rabbit IgG (1:500, ab150077, Abcam) were finally used to detect the expression of PFKFB3 and Iba-1. Using a Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany), images were taken at 20 × magnification. In each image, the total count of PFKFB3 and Iba-1-positive cells was estimated in an attempt to determine the total number of activated microglia.

Y-maze

The Y-maze (Sansbio, JiangSu province) was a modified version of a previously described apparatus [25]. Mice were initially placed in one arm, and the sequence and number of arm entries were recorded with ANY-maze (Stoelting CO., Wood Dale, IL, USA). Each mouse was placed at the end of one arm after 24 hr of injecting LPS and 2-GBI and allowed to explore the apparatus freely for 8 minutes. The results of the test performed were recorded and the following parameters like spontaneous alteration performance (SAP), alternate arm return (AAR) and same arm return (SAR) were determined. SAP is the main criteria that tests spatial learning and memory, which was defined as actual alternation/possible alternation. AAR and SAR in the Y-maze (AAR = alternate arm returns/total arm entries × 100, SAR = same arm return/total arm entries × 100) were used as indicators of memory impairment.

Data processing

Statistical multiple group comparisons were performed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test in the GraphPad Prism software, v8.0 (CA, USA) and
results were reported as mean ± standard error (SEM). A value of $P < 0.05$ was used to indicate significant difference.

**Results**

**2-GBI alleviates the LPS-induced inflammatory response in microglia cells**

We evaluated the impact of 2-GBI, a known Hv1 inhibitor in modulation of neuroinflammatory response. For this purpose, primary microglia and BV2 cells were treated with 200 µM 2-GBI and LPS (50 ng/mL). We first analyzed LPS-induced generation of inflammatory modulators. The results presented that 2-GBI inhibited LPS-induced NO production (Fig. 1A and B), protein expressions of iNOS, COX2, TNFα, and IL-1β in primary microglia and BV2 cells (Fig. 1C-E).

**2-GBI attenuates the LPS-induced aerobic glycolysis in microglial cells**

Glycolytic reprogramming plays an important role in polarization of inflammatory cells. In 2-GBI-treated cells, the LPS-induced glycolysis-related proteins of HK2 and PFKFB3 were decreased (Fig. 2A-B). Therefore, we examined whether Hv1-mediated aerobic glycolysis and enhanced inflammatory responses were dependent on HIF1α expression. As shown in Fig. 2A-B, protein expression of HIF1α were elevated in response to LPS stimulation and mitigated in response to 2-GBI. Next, 2-GBI-treated BV2 cells and primary microglia unveiled the lactate levels for LPS-activated status undergoing a marked decline (Fig. 2C). In addition, as an indirect indicator of lactate generation and improved glycolytic metabolism ECAR was seen to be elevated in LPS-treated primary microglia and BV2 cells, and 2-GBI significantly inhibited LPS-induced glycolytic metabolism in microglial cells following 4 hrs of LPS treatment (Fig. 2D). As shown in Fig. 2D, addition of 2-GBI was found to have pronounced inhibitory effect on the basal OCR, ATP-linked OCR, maximal respiration and SRC of microglial cells and no increasing effect on ECAR. ATP-linked OCR and SRC were noticeably increased by LPS (50 ng/mL) exposure, while 2-GBI treatment effectively rescued this induction. The OCR was significantly lower in 2-GBI/LPS group than the LPS group in microglial cells following 4 hrs of LPS treatment. It is clearly evident from the data that mitochondrial respiration and aerobic glycolysis in microglial cells was inhibited by Hv1.

**The anti-inflammatory effect of Hv1 depends on HIF1α**

To further explore linkage between decreased expression of HIF1α and aerobic glycolysis due to Hv1 inhibitor, we assessed PFKFB3 and HK2 levels in BV2 cells induced HIF1α by CoCl2 treatment (Fig. 3). As shown in Fig. 3A-D, the protein level of HIF1α, HK2 and PFKFB3 in microglia were significantly increased after CoCl2 treatment, while this upregulation effects of CoCl2 were relieved by 2-GBI (Fig. 3A-D).
Effects of 2-GBI on PI3K/AKT/mTOR and ERK1/2 activation in microglia

The PI3K/AKT and ERK1/2 pathways are involved in the regulation of HIF1α. We investigated whether 2-GBI could affect PI3K/AKT and ERK1/2 phosphorylation in microglia. As shown in Fig. 4A-C, 2-GBI significantly decreased the phosphorylation of PI3K/AKT/mTOR and ERK1/2 expression in BV2 cells and primary microglia after 4 hours of LPS exposure. These results suggest that the PI3K/AKT and ERK1/2 pathway have a role in LPS-induced microglial activation, and that 2-GBI reduces LPS-induced inflammatory responses by PI3K/AKT/HIF1α and ERK1/2/HIF1α signaling. An increasing body of evidence shows that activated microglial cells generated intracellular ROS and play a significant role in HIF1α-mediated enhancement of the inflammatory response [26]. Hence, the impact of Hv1 on the production of intracellular ROS was determined using ROS-sensitive indicators DCFH-DA. 2-GBI-treated BV2 cells manifested a decrease in intracellular ROS for LPS-activated status (Fig. 4D).

2-GBI Regulates the Expression of Inflammatory Mediators in Neurons/Glial Cells Co-cultures Submitted to LPS Stimulus

To study the neuroprotective effects of 2-GBI, we constructed a noncontact co-culture system of BV2 and PC12 cells by the transwell system to mimic the growth environment of microglia and dopaminergic neurons. First, we measured the viability of PC12 cells at 24 hr after co-culture with LPS-activated BV2 using CCK-8. The results showed that the viability of PC12 cells after co-culture with LPS-activated BV2 decreased significantly at 24 hours (Fig. 5A). Next, we observed that 2-GBI significantly reversed PC12 cell death (Fig. 5A). Furthermore, western blot results showed that LPS-induced BV2 upregulated cleaved-caspase-3 levels and downregulated synaptophysin levels in PC12 cells, resulting in a significant inflammation-mediated neurotoxicity in PC12 cells. In contrast, 2-GBI significantly protect against inflammation by improving the levels of cleaved-caspase-3 and synaptophysin in PC12 cells (Fig. 5B-F). Meanwhile, TUNEL results also showed that BV2 pre-treated with 2-GBI decreased PC12 cell apoptosis (Fig. 5G).

2-GBI suppresses LPS-induced brain inflammation in mouse

To study the effect of Hv1 inhibitor on LPS-induced memory impairment and neurotoxicity in vivo, adult C57BL/6J mice were treated with 5 mg/kg LPS intraperitoneally with or without adminstration of 2-GBI (1 mg/kg, intraperitoneally). The levels of TNFα, IL-1β and iNOS underwent a marked increase in the mouse hippocampus 6hrs following the LPS challenge. Nevertheless, the elevation of these cytokines was averted by 2-GBI (Fig. 6A and B). Furthermore, increased PFKFB3 positive microglia were detected in the CA1 and DG regions of the LPS-treated mice, and 2-GBI alleviated LPS-induced glycolysis in the hippocampus (Fig. 6C-E).
We carried out a Y-maze behavioral test to assess spatial working memory affected by the Hv1 inhibitor. Alternation of arm entries in a Y-maze is driven by an instinct to visit a novel place and requires the animal to remember which arms it entered in its immediately previous exploration. During the 8 min test session in the Y-maze, LPS treatment significantly reduced the probability of SAP to 52% compared to 62% in vehicle-treated control mice (Fig. 7A). 2-GBI prevented the decrease of SAP caused by LPS administration (Fig. 7A). Correspondingly, LPS significantly increased the probability of SAR, while 2-GBI reversed the cognition deficits (Fig. 7B). AAR did not produce a difference among groups in the Y-maze test (Fig. S1). These results suggest that 2-GBI reverses the spatial working memory impairment caused by LPS-induced neuroinflammation.

Discussion

The current work reveals evidence that microglial Hv1 acts as an immunometabolic regulator that controls inflammatory cytokine production. In both primary and BV2 microglia, Hv1 inhibitor impairs LPS-induced pro-inflammatory mediator synthesis and aerobic glycolysis. Mechanistic studies identified that PI3K/AKT/HIF1α and ROS/HIF1α signaling pathways were involved in Hv1 mediated aerobic glycolysis. We further demonstrated that Hv1 inhibitor was able to suppress the inflammatory responses and the altered metabolic processes caused by LPS insult in both vitro vivo. Systematic administration of Hv1 inhibitor also improved LPS-induced deficits in recognition memory in mice.

Hv1, having a functional expression within microglia, aids in NOX-dependent generation of ROS and controls intracellular pH [14]. Recent studies have revealed that a deficiency of Hv1 was able to weaken the disruption of white matter integrity induced by bilateral common carotid artery stenosis [27], alleviate neuronal apoptosis and neuronal pyroptosis following SCI [28], and reduce LPC-mediated myelin damage [29]. Our group has previously found that Hv1 upregulation in the aged brain exaggerates postoperative neuroinflammatory responses after peripheral tibia fracture surgery [15]. In the current work, we further confirmed previous finding that Hv1 plays a critical role in microglia-mediated neuroinflammation and emerges as a potential therapeutic target for neuroprotection.

Evidence suggests an essential involvement of metabolic reprogramming in regulating the inherent inflammatory response [30]. In response to immune challenge, cells choose to use glycolysis instead of mitochondrial catabolic pathways for the conservation and generation of metabolic resources. As a consequence of this switch, there is an increase in lactate production and glucose uptake with activation of the pentose phosphate pathway (PPP) and a simultaneous decrease in oxygen consumption by mitochondria [31]. Succinate, the Krebs cycle intermediate, regulates HIF1α in M1 macrophages to drive a sustained production of IL-1β [32]. The addition of 2-DG, a glycolysis inhibitor, blocked the immunometabolic reprogramming of microglia resulting from an LPS-induced rise in aerobic glycolysis [12]. A high or moderate dose of LPS-induced cells became more dependent on glycolysis than mitochondrial respiration [33]. The molecular mechanisms behind this response is not known, but it has been proposed that the energy depletion elicits mitochondrial damage. Without LPS treatment, impaired Hv1 activity by 2-GBI results in significantly reduced OCR. The current study showed that Hv1 was found
to have pronounced down-regulatory effect on the OCR of microglia and no increasing effect on ECAR. Nevertheless, LPS pushed aerobic glycolysis and generation of the proinflammatory cytokine in microglial cells, and 2-GBI effectively attenuated LPS-induced glycolysis in microglial cells, thereby implying that Hv1 play an important role in control of microglial activation via metabolic reprogramming.

The switch to glycolysis is promoted by the transcription factor HIF1α so that these cells can continue to generate ATP. HIF1α aids in this metabolic switch by bonding with the hypoxia response elements within target genes, such as glycolytic enzymes and the glucose transporter GLUT1 [34]. HIF1α also increments the expression of glycolysis-related genes PFKFB3, a major driver of glycolysis by its ability to synthesize fructose-2,6-bisphosphate [35]. When HIF1α is absent, the cellular ATP levels are greatly reduced [36]. In the current study, we detected an elevation of HIF1α expression in LPS-stimulated microglial cells. Inhibition of HIF1α expression in microglia attenuated LPS-induced aerobic glycolysis as well as the inflammatory response, thereby implying that the regulating microglial metabolic reprogramming and subsequent inflammatory responses by Hv1 were dependent upon the expression of HIF1α.

In microglia, ROS are generated primarily by NOX2 [37]. It has been suggested that Hv1 channels are an essential regulator of ROS production in microglia by counteracting the charge imbalance caused by the activation of NADPH oxidase [38]. In the current study, we found that the Hv1 inhibition suppressed ROS expression and abolished enhancement of the inflammatory response in LPS stimulated microglial cells. There is evidence that ROS act as second messengers to propagate microglial immune activation by influencing multiple key signaling pathways, including PI3K/AKT/mTOR and MAPKs [38]. Phosphorylated mTOR and ERK1/2 were suggested to increase the expression of HIF1α [39–40]. We found that 2-GBI inhibited PI3K/AKT/mTOR and ERK1/2 phosphorylation. Thus, these data indicated that Hv1 regulates HIF1α expression and metabolic reprogramming through the PI3K/AKT/HIF1α and ROS/HIF1α mediated pathway.

This work investigates the effect of a prototypical Hv1 inhibitor (2-GBI) on LPS induced neuroinflammation both in vitro and in vivo. 2-GBI inhibits Hv1 proton conduction by binding to the VSD from its intracellular side [41]. We found that 2-GBI effectively ameliorated neuroinflammation through inhibition of HIFα-mediated aerobic glycolysis in cultured microglia. Systematic administration of 2-GBI facilitated the recovery of recognition memory in mice challenged with LPS. The 2-GBI associated cognition recovery from LPS stimulation was paralleled by reduced levels of IL-1β, TNFα and iNOS in the hippocampus. Our finding suggested that Hv1 inhibitors can be considered as potential pharmacological treatments for diseases caused by Hv1 hyperactivity in future.

**Conclusions**

In summary, the present study provides the evidence showing that Hv1 modulates microglial activation via regulation of metabolic reprogramming. HIF1α is essential for Hv1-mediated inflammation, and activation of the PI3K/AKT/HIF1α as well as ROS/HIF1α pathway are involved in Hv1-mediated glycolytic reprogramming. The observations that pharmacological targeting of microglial Hv1 channels can affect
hippocampal related cognitive function and neuroinflammation in mice are particularly interesting and interfering with this pathway may provide a promising therapeutic option for neuroinflammatory diseases.

**Abbreviations**

LPS  
lipopolysaccharide  
ANOVA  
Analysis of variance  
SAE  
sepsis-associated encephalopathy  
(TLR)-4  
toll-like receptor-4  
CNS  
Central nervous system  
IL  
interleukin  
TNFα  
tumor necrosis factorα  
TLR  
Toll-like receptor  
2-GBI  
2-guanidinobenzimidazole  
COX2  
cyclooxygenase-2  
OCR  
oxygen consumption rate  
DMEM  
Dulbecco’s modified Eagle’s medium  
FBS  
fetal bovine serum  
PFKFB3  
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3  
HK2  
hexokinase 2  
HIF1α  
hypoxia-inducible factor 1α  
ECAR  
extracellular acidification rates
Arg-2
glutinase-2
GLUT1
glucose transporter 1
PPP
pentose phosphate pathway
2-DG
2-deoxyglucose
PBS
phosphate buffer saline
siRNA
small interfering RNA
SRC
spare respiratory capacity
GFP
green fluorescent protein
Ym1
Chitinase-3-like protein 3
NOX2
NADPH oxidase 2.

Declarations

Acknowledgements

Not applicable.

Availability of supporting data

Not applicable.

Authors’ contributions

LS contributed to the design of the study, performed transcript, behavioral and histological analyses, analyzed the experiments, wrote the manuscript; TL designed the experiment, analyzed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the Natural Science Foundation of Guangdong Province 2021A1515011601 (LS), Shenzhen Science and Technology Foundation JCYJ20190807155005597 (LS).

Availability of data and materials
Ethics approval and consent to participate

All animal procedures were approved by the Institute of Animal Care Committee of Peking University Shenzhen Hospital, China.

Consent for publication

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

References

1. Amor S, Peferoen LA, Vogel DY, Breur M, van der Valk P, Baker D, van Noort JM. Inflammation in neurodegenerative diseases—an update. Immunology. 2014;142:151-166.

2. ElAli A, Rives S. Microglia in Alzheimer's disease: a multifaceted relationship. Brain Behav Immun. 2016;55:138-150.

3. Dubbelaar ML, Kracht L, Eggen BJ, Boddeke EW. The kaleidoscope of microglial phenotypes. Front Immunol. 2018;9:1753.

4. Badimon A, Strasburger HJ, Ayata P, Chen X, Nair A, Ikekami A, Hwang P, Chan AT, Graves SM, Uweru JO, Ledderose C, Kutlu MG, Wheeler MA, Kahan A, Ishikawa M, Wang YC, E. Loh YH, Jiang JX, Surmeier DJ, Robson SC, Junger WG, Sebra R, Calipari ES, Kenny PJ, Eyo UB, Colonna M, Quintana FJ, Wake H, Gradinaru V, Schaefer, A. Negative feedback control of neuronal activity by microglia. Nature. 2020;586:417-423.

5. Belarbi K, Jopson T, Tweedie D, Arellano C, Luo W, Greig NH, Rosi S. TNF-α protein synthesis inhibitor restores neuronal function and reverses cognitive deficits induced by chronic neuroinflammation. J Neuroinflammation 2012;9:1-13.

6. Donat CK, Scott G, Gentleman SM, Sastre M. Microglial activation in traumatic brain injury. Front Aging Neurosci. 2017; 9:208.

7. Gu R, Zhang F, Chen G, Han C, Liu J, Ren Z, Zhu Y, Waddington JL, Zheng LT, Zhen, X. Clk1 deficiency promotes neuroinflammation and subsequent dopaminergic cell death through regulation of microglial
metabolic reprogramming. Brain Behav Immun. 2017;60: 206-219.

8. Baik SH, Kang S, Lee W, Choi H, Chung S, Kim JI, Mook-Jung I. A breakdown in metabolic reprogramming causes microglia dysfunction in Alzheimer's disease. Cell Metab. 2019;30:493-507.

9. Du L, Lin L, Li Q, Liu K., Huang, Y., Wang, X., Cao K, Chen X, Cao W, Li F, Shao C, Wang Y, Shi Y. IGF-2 preprograms maturing macrophages to acquire oxidative phosphorylation-dependent anti-inflammatory properties. Cell Metab. 2019;29:1363-1375.

10. Amici SA, Dong J, Guerau-de-Arellano M. Molecular mechanisms modulating the phenotype of macrophages and microglia. Front Immunol. 2017;8:1520.

11. Liao ST, Han C, Xu DQ, Fu XW, Wang JS, Kong LY. 4-Octyl itaconate inhibits aerobic glycolysis by targeting GAPDH to exert anti-inflammatory effects. Nat Commun. 2019;10:1-11.

12. York EM, Zhang J, Choi HB, MacVicar BA. Neuroinflammatory inhibition of synaptic long-term potentiation requires immunometabolic reprogramming of microglia. Glia. 2021;69: 567-578.

13. Kawai T, Kayama K, Tatsumi S, Akter S, Miyawaki N, Okochi Y, Abe M, Sakimura K, Yamamoto H, Kihara S,Okamura Y. Regulation of hepatic oxidative stress by voltage-gated proton channels (Hv1/VSOP) in Kupffer cells and its potential relationship with glucose metabolism. FASEB J. 2020;34:15805-15821.

14. Qiu F, Chamberlin A, Watkins BM, Ionescu A, Perez ME, Barro-Soria R, Gonzalez C, Noskov SY, Larsson HP. Molecular mechanism of Zn2+ inhibition of a voltage-gated proton channel. Proc Natl Acad Sci U S A. 2016;113:E5962–e5971.

15. Zhang ZJ, Zheng XX, Zhang XY, Zhang Y, Huang BY, Luo T. Aging alters Hv1-mediated microglial polarization and enhances neuroinflammation after peripheral surgery. CNS Neurosci Ther. 2020;26:374-384.

16. Wu LJ, Wu G, Sharif MRA, Baker A, Jia Y, Fahey FH, Luo HR, Feener EP, Clapham, DE. The voltage-gated proton channel Hv1 enhances brain damage from ischemic stroke. Nat Neurosci. 2012;15(4):565-573.

17. Li X, Yu Z, Zong W, Chen P, Li J, Wang M, Ding F, Xie M, Wang W, Luo, X. Deficiency of the microglial Hv1 proton channel attenuates neuronal pyroptosis and inhibits inflammatory reaction after spinal cord injury. J Neuroinflammation. 2020;17:263.

18. He J, Ritzel RM, Wu J. Functions and Mechanisms of the Voltage-Gated Proton Channel Hv1 in Brain and Spinal Cord Injury. Front Cell Neurosci. 2021;15:112.

19. Ritzel RM, He J, Li Y, Cao T, Khan N, Shim B, Sabirzhanov B, Aubrecht T, Stoica BA, Faden AI, Wu LJ, Wu, J. Proton extrusion during oxidative burst in microglia exacerbates pathological acidosis following
traumatic brain injury. Glia. 2021;69:746–764.

20. Wang Y, Lawson MA, Dantzer R, Kelley KW. LPS-induced indoleamine 2, 3-dioxygenase is regulated in an interferon-γ-independent manner by a JNK signaling pathway in primary murine microglia. Brain Behav Immun. 2010;24(2):201-209.

21. Sun L, Ma W, Gao W, Xing Y, Chen L, Xia Z, Zhang Z, Dai Z. Propofol directly induces caspase-1-dependent macrophage pyroptosis through the NLRP3-ASC inflammasome. Cell Death Dis. 2019;10(8):1-14.

22. Yin Z, Han Z, Hu T, Zhang S, Ge X, Huang S, Wang L, Yu J, Li W, Wang Y, Li D, Zhao J, Wang Y, Zuo Y, Li Y, Kong X, Chen F, Lei P. Neuron-derived exosomes with high miR-21-5p expression promoted polarization of M1 microglia in culture. Brain Behav Immun. 2020;83:270-282.

23. Cai J, Xu D, Bai X, Pan R, Wang B, Sun S, Chen R, Sun J, Huang, Y. Curcumin mitigates cerebral vasospasm and early brain injury following subarachnoid hemorrhage via inhibiting cerebral inflammation. Brain Behav. 2017;7(9):e00790.

24. Depino AM. Early prenatal exposure to LPS results in anxiety-and depression-related behaviors in adulthood. Neuroscience. 2015;299:56-65.

25. Zhong L, Jiang X, Zhu Z, Qin H, Dinkins MB, Kong JN, Leanhart S, Wang R, Elsherbin A, Bieberich E, Zhao Y, Wang, G. Lipid transporter Spns2 promotes microglia pro-inflammatory activation in response to amyloid-beta peptide. Glia. 2019;67(3):498-511.

26. Chuang DY, Simonyi A, Kotzbauer PT, Gu Z, Sun GY. Cytosolic phospholipase A 2 plays a crucial role in ROS/NO signaling during microglial activation through the lipoxygenase pathway. J Neuroinflammation. 2015;12:1-20.

27. Yu Y, Luo X, Li C, Ding F, Wang M, Xie M, Yu Z, Ransom BR, Wang W. Microglial Hv1 proton channels promote white matter injuries after chronic hypoperfusion in mice. J Neurochem. 2020;152:350-367.

28. Li X, Yu Z, Zong W, Chen P, Li J, Wang M, Ding F, Xie M, Wang W, Luo, X. Deficiency of the microglial Hv1 proton channel attenuates neuronal pyroptosis and inhibits inflammatory reaction after spinal cord injury. J Neuroinflammation. 2020;17:263.

29. Chen M, Yang LL, Hu ZW, Qin C, Zhou LQ, Duan YL, Bosco DB, Wu LJ, Zhan KB, Xu SB, Tian DS. Deficiency of microglial Hv1 channel is associated with activation of autophagic pathway and ROS production in LPC-induced demyelination mouse model. J Neuroinflammation. 2020;17:333.

30. Kelly B, O’neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res. 2015;25:771-784.
31. Lauro C, Limatola C. Metabolic reprogramming of microglia in the regulation of the innate inflammatory response. Front Immunol. 2020;11:493.

32. Orihuela R, McPherson CA, Harry GJ. Microglial M1/M2 polarization and metabolic states. Brit J Pharmacol. 2016;173(4):649-665.

33. Nair S, Sobotka KS, Joshi P, Gressens P, Fleiss B, Thornton C, Mallard C, Hagberg H. Lipopolysaccharide-induced alteration of mitochondrial morphology induces a metabolic shift in microglia modulating the inflammatory response in vitro and in vivo. Glia. 2019; 67(6):1047-1061.

34. Bernier LP, York EM, MacVicar BA. Immunometabolism in the Brain: How Metabolism Shapes Microglial Function. Trends Neurosci. 2020;43:854-869.

35. Wang Z, Kong L, Tan S, Zhang Y, Song X, Wang T, Lin Q, Wu Z, Xiang P, Li C, Liang X, Ma, C. Zhx2 accelerates sepsis by promoting macrophage glycolysis via Pfkfb3. J Immunol 2020;204(8):2232-2241.

36. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase V, Jaenisch R, Corr M, Nizet V, Firestein G, Gerber H, Ferrara N, Johnson R, Johnson, R. HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell 2003;112:645–657.

37. Dohi K, Ohtaki H, Nakamachi T, Yofu S, Satoh K, Miyamoto K, Song D, Tsunawaki S, Shioda S, Aruga, T. Gp91 phox (NOX2) in classically activated microglia exacerbates traumatic brain injury. J Neuroinflammation. 2010;7:1-11.

38. Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W. ROS and ROS-mediated cellular signaling. Oxid Med Cell Longev. 2016;2016:18.

39. Jones NM, Bergeron M. Hypoxia-induced ischemic tolerance in neonatal rat brain involves enhanced ERK1/2 signaling. J Neurochem. 2004;89:157-167.

40. Land SC, Tee AR. Hypoxia-inducible factor 1α is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J Biol Chem. 2007;282(28): 20534-20543.

41. Hong L, Kim IH, Tombola F. Molecular determinants of Hv1 proton channel inhibition by guanidine derivatives. P Natl Acad Sci USA. 2014;111:9971-9976.

**Figures**

**Figure 1**

2-guanidobenzimidazole (2-GBI), a specific HVCN1-channel blocker, leads to suppression of LPS-stimulated inflammation via regulation of primary microglial metabolic reprogramming. 2-GBI alleviated
the lipopolysaccharide (LPS)-inducible NOS (iNOS) expression in cultured primary microglia (A) and BV2 cells (B). Cells were stimulated with LPS (50 ng/mL). After 12 h, the amount of NO in the medium was measured by Griess reagent. Data are presented as mean ± SEM for 6 independent experiments. (C) 2-GBI inhibited the enhanced expression of inflammation in the primary microglia cells after 4 and 6 hr of LPS exposure. (D) 2-GBI alleviated the enhanced expression of inflammation in BV2 cells after 4 hr of LPS exposure. (E) Band intensity was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. All the data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown.

**Figure 2**

2-GBI leads to suppression of LPS-stimulated inflammation via regulation of microglial metabolic reprogramming in cultured primary microglia and BV2 cells. (A) 2-GBI inhibited the enhanced expression of inflammation via glycolytic metabolism in cultured primary microglia and BV2 cells after 4 hr of LPS exposure. (B) Band intensity in BV2 cells was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. (C) 2-GBI rescued LPS-induced cellular L-lactate increase in cultured primary microglia and BV2 cells. The data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown. (D) 2-GBI treatment reversed the metabolic shift in microglia. Treatment of LPS resulted in increased spare respiratory capacity and ATP-linked OCR at 4 hr, whereas inhibition of Hv1 by 2-GBI decreased basal OCR, ATP-linked OCR, maximal respiration and spare respiratory capacity at 4 hr compared with 50 ng/mL LPS exposure in cultured primary microglia and BV2 cells. 2-GBI normalized LPS-induced increased ECAR dependent glycolysis, glycolytic capacity and glycolytic reserve in cultured primary microglia and BV2 cells. OCR and ECAR measured are expressed in bar graph format as the mean ± SD, n = 6. *, P < 0.05 compared to Ctrl. #, P < 0.05 compared to LPS group.

Fig. 3. 2-GBI reverses HIF1α and glycolytic enzymes production in CoCl2-treated microglia. BV2 cells were pretreated with 200 μM CoCl2 for 24 hrs, then treated with 200 μM 2-GBI for 9 h. (A) The expression of HIF1α, PFKFB3, and HK2 was determined by western blotting. (B-D) Band intensity was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. All the data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown.

**Figure 3**

2-GBI results in decreased production of reactive oxygen species in BV2 cells. (A-B) 2-GBI inhibited the enhanced expression of glycolytic metabolism via mTOR, PI3K/AKT and ERK1/2 signaling pathway in
cultured primary microglia and BV2 cells after 4 hours of LPS exposure. (C) Band intensity in BV2 cells was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. All the data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown. (D) The BV2 cells with or without 2-GBI were incubated with LPS (50 ng/mL) for 4 hr and intracellular ROS levels were measured by DCFH-DA. Fluorescence assay were detected in BV2. Data are presented as mean ± SEM for at least 6 independent experiments.

Figure 4

The mechanisms of inhibition of Hv1 attenuates LPS-induced microglial inflammation and consequent neuron death. (A) Effect of BV2-conditioned medium on PC12 cell viability. After treatment of the conditioned medium for 24 h, cell viability was measured by the CCK-8 assay. (B) Protein expressions of Caspase-3, PSD95 and synaptophysin in PC12 were determined by western blotting. (C-F) Band intensity was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. (G) TUNEL assay detected apoptosis in PC12. Scale bar = 100 μm. All the data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown.

Figure 5

The mechanisms of inhibition of Hv1 attenuates LPS-induced microglial inflammation and consequent neuron death. (A) Effect of BV2-conditioned medium on PC12 cell viability. After treatment of the conditioned medium for 24 h, cell viability was measured by the CCK-8 assay. (B) Protein expressions of Caspase-3, PSD95 and synaptophysin in PC12 were determined by western blotting. (C-F) Band intensity was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. (G) TUNEL assay detected apoptosis in PC12. Scale bar = 100 μm. All the data are expressed as means ± SEM and were analyzed by two-way ANOVA with the Tukey’s post hoc test. N = 6 independent measurements. *P < 0.05; **P < 0.01 for comparisons shown.

Figure 6

2-GBI suppress the inflammatory response in an LPS-induced neuroinflammation mouse model. Wild type (WT) mice received intraperitoneal injections of LPS (5 mg/kg), 2-GBI (1 mg/kg) or saline (NS) for 1 days as described in Methods. Mice were sacrificed and brains collected after 6 hours of injecting LPS and 2-GBI. (A) 2-GBI treatment suppressed the expressions of proinflammatory factors in the
hippocampus of LPS-treated mice. (B) Band intensity was quantified by ImageJ software, and the values of target protein were normalised to that of β-actin. (C) Immunostaining for PFKFB3 and Iba-1 in the CA1 and DG regions. Bar: 50 μm. (D) Quantification of Iba1 positive cells/mm² in the CA1 and DG regions. (E) Ratio of PFKFB3 positive microglia in the CA1 and DG regions. Data are presented as mean ± SEM. Significance was determined by two-way ANOVA with Tukey’s test; Significance scores are * for $P < 0.05$, ** for $P < 0.01$.

Figure 7

2-GBI ameliorated LPS-induced cognition in vivo. Following 24 h of injecting LPS and 2-GBI, the animals were subjected to Y-maze to test spatial working memory (n=8). (A-B) 2-GBI ameliorated the memory decline caused by LPS shown by: improved Spontaneous alternation performance (SAP) and reduced same arm return (SAR). SAP is defined as the percentage of triads that an animal goes into three different arms of the Y-maze in a triad entry and SAR as the percentage of an animal returning to the same arm in any consecutive entries in a triad entry. Data are presented as mean ± SEM. Significance was determined by two-way ANOVA with Tukey’s test; Significance scores are * for $P < 0.05$, ** for $P < 0.01$.

Supplementary Files

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