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Nicorandil Inhibits Inflammasome Activation and Toll-Like Receptor-4 Signal Transduction to Protect against Oxygen–Glucose Deprivation-Induced Inflammation in BV-2 Cells

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SUMMARY

Background and purpose: Our previous studies have demonstrated adenosine triphosphate-sensitive potassium channel (K_{ATP} channel) openers could protect against inflammatory response in brain disease, but little is known about the mechanisms involved in K_{ATP} channel openers inhibiting neuroinflammation. **Methods and results:** In the present study, we found that oxygen–glucose deprivation (OGD) resulted in BV-2 cells activation, significantly increased tumor necrosis factor-alpha and interleukin-1beta (IL-1β) levels, accompanied by downregulating Kir6.1 subunit. Pretreatment with nicorandil, a K_{ATP} channel opener, could attenuate OGD-induced BV-2 cells activation and inhibit pro-inflammatory factors release. Further study demonstrated that OGD activated Toll-like receptor-4 (TLR4) signaling pathway and NOD-like receptor pyrin domain containing three inflammasome, thereby increased IL-1β production. Pretreatment with nicorandil could reverse the two pathways involved in IL-1β production. **Conclusions:** Our findings reveal that K_{ATP} channel openers could protect against OGD-induced neuroinflammation via inhibiting inflammasome activation and TLR4 signal transduction.

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The first two authors contributed equally to this work.

Introduction

Stroke is the third leading cause of death, which is only next to the heart disease and cancer. At present, the only globally approved treatment for stroke is tissue plasminogen activator (tPA). However, this treatment only suits for <5% patients, owing to its narrow time-dependent therapy and complications [1]. It is urgent to reveal the pathophysiological mechanisms of stroke, which is essential for drug development. Increasing evidence suggests that post-ischemic inflammation is involved in the progress of ischemic cascade, from acute brain damage to tissue repair [2]. At the early stage of ischemic stroke onset, microglia is activated rapidly and produces lots of pro-inflammatory mediators, which trigger parenchymal inflammation and recruit infiltrating immune cells from peripheral system [3]. Microglia is the resident immune cells in the brain, which is also the major resource of inflammatory cytokines at the initial stage of brain ischemia [4]. Despite the fact that there are intensive studies of post-ischemic inflammation, the molecular mechanisms that activate resident microglia and drive the production of inflammatory cytokines remain unclear. Interleukin-1beta (IL-1β) is one of the most potent mediators in inflammation, which is produced from an inactive precursor (pro-IL-1β) by immune cells such as macrophages or microglia during diseases or after injury [5,6]. Caspase-1 is the key protease required for cleaving pro-IL-1β into IL-1β, its activation is controlled by its recruitment to multimolecular scaffolds named inflammasome [7]. NOD-like receptor pyrin domain containing three (NLRP3) is implicated as a sensor of sterile injury [8]. Whether production of IL-1β in response to oxygen–glucose deprivation (OGD) is due to inflammasome activation remains unclear.

Adenosine triphosphate (ATP)-sensitive potassium channels (K_{ATP} channels) couple cellular energetic metabolism with electric activity [9]. Accumulating evidence indicates Kir6.1-contained K_{ATP} channels mainly express in glia [10–12]. Our previous study has demonstrated that Kir6.1-contained K_{ATP}

channels are involved in brain ischemic injury, Kir6.1 knockdown aggravates ischemic injury via augmenting reactive glia and inflammatory responses [13]. In addition, other studies also elucidate that Kir6.1-contained K_{ATP} channels are pivotal targets for regulating inflammatory responses, which is implicated in disease process [14,15]. But little is known about whether and how K_{ATP} channels are concerned with post-ischemic inflammation and microglial responses in stroke.

The present study was to determine the impacts of K_{ATP} channel opener nicorandil on OGD-induced inflammatory responses in BV-2 cell lines and clarify the involved mechanisms. Our work showed that OGD could induce production of IL-1β, which was involved with upregulation of Toll-like receptor 4 (TLR4), NLRP3, and cleaved caspase-1. However, pretreatment with nicorandil could reverse the above phenomena and decrease production of IL-1β. These results suggest that K_{ATP} channels are involved in regulating the production of IL-1β in microglia after OGD injury.

Materials and Methods

Cell Cultures and Treatment

BV-2 cells were generated by immortalizing primary murine microglial cells with a v-raf/v-myc oncogene carrying retrovirus, as seen in previous studies [14]. BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (10%) (GIBCO, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 mg/L). The cells were gently suspended in DMEM and plated in six-well plates at a density of 1×10^5 cells/mL. In all experiments, cells were pretreated with nicorandil (a K_{ATP} channel opener) (TOCRIS, Bristol, UK) and PNU37883 (a selective Kir6.1 blocker) (TOCRIS) for 1 h before OGD. Control samples contained the same volume of vehicle. The experimental groups were divided as follows: (1) control, (2) PNU37883 (20 μM), (3) nicorandil (10 μM), (4) OGD, (5) OGD + PNU37883 (20 μM), and (6) OGD + nicorandil (10 μM).

Oxygen–Glucose Deprivation

After washing twice, BV-2 cells were immersed in 1 mL deoxygenated custom DMEM medium without glucose (GIBCO), oxygen removed, and the six-well plates were placed inside an incubator (Thermo scientific, Waltham, MA, USA) for 1 h with a pre-mixed gas (1% O_2, 94% N_2, 5% CO_2). Control cultures were treated similarly but with normoxic DMEM supplemented with 3 mM D-glucose (GIBCO) in a normoxic incubator.

Cell Viability Analysis

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect cell viability as Zhou et al. [11] described before. BV-2 cells were cultured in 96-well plates at a density of 5×10^4 cells per well. After different time-course (0.5 h/1 h/1.5 h) of OGD and reoxygenation 24 h, the MTT was dissolved in DMEM medium as a final concentration of 0.5 mg/mL and incubated at 37°C for 4 h. Then, we replaced the medium with 150 μL dimethyl sulfoxide (DMSO) to dissolve the formazan product. The absorbance of each well was obtained using a Dynatech MR5000 plate counter (Dynatech Corp., Burlington, MA, USA) at a test wavelength of 570 nm with a reference wavelength of 630 nm. All data are expressed as mean ± SEM of three independent experiments.

Lactate Dehydrogenase Release

After different time-course (0.5 h/1 h/1.5 h) of OGD and reoxygenation 24 h, lactate dehydrogenase (LDH) release in culture medium was measured by using the LDH diagnostic kit (Jiancheng Bioengineering, Nanjing, China), according to manufacturer's instructions. LDH activity was calculated by measuring absorbance at 490 nm. All the detail experimental procedures were as described before by Xie et al. [16].

Western Blotting

The cytosolic protein in samples was extracted according to the KEYGEN protein extraction kit (KeyGen Biotech. Co. Ltd., Nanjing, China). Protein concentrations were determined using the Micro BCA Kit (Pierce Biotechnology, Rockford, IL, USA). The supernatants (50 μg protein) were separated by Tris–glycine SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA) with the electrophoretic transfer system (Trans-blot Semi-dry Transfer Cell; Bio-Rad, Hercules, CA, USA) and blocked with 10% nonfat dry milk in Tris–HCl buffer saline (TBS, pH 7.4) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Then, the PVDF membranes were incubated with primary antibody against Kir6.1 (1:200; Santa Cruz, Dallas, TX, USA), SUR1 (1:100; Santa Cruz), SUR2 (1:100; Santa Cruz), Caspase-1 (1:400; Santa Cruz), TLR4 (1:400; Santa Cruz), pIKKa/β (1:800; CST, Boston, MA, USA), NLRP3 (1:200; Santa Cruz), and IL-1β (R&D Systems, Minneapolis, MN, USA) overnight at 4°C. After being washed in TBS-T, the membranes were incubated with corresponding secondary antibody for 1 h at room temperature. Finally, visualization of the signal was performed by enhanced chemiluminescence (Ultra-Lum, Claremont, CA, USA). Quantification of bands was made by scanned densitometric analysis and Image J analysis system.

Enzyme-Linked Immunosorbent Assay

After 24-h reoxygenation, we collected the medium from BV-2 cells. Measurement of key inflammatory cytokines (TNF-a, IL-1β, and IL-10) released into the culture supernatant was performed using specific enzyme-linked immunosorbent assays (ELISAs) (TNF-a and IL-10 were obtained from R&D Systems; IL-1β was obtained from Biogot technology co., Ltd., Nanjing, China) according to manufacturers guidelines.

Statistical Analysis

Data are shown as mean ± SEM. Unless stated otherwise, we carried out all statistical quantitative assessments in a blinded manner. For two groups paired *t*-test (two-tailed), for three or more groups one-way or two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls tests. Differences were considered significant for $P < 0.05$.

Results

OGD Downregulates Protein Expression of Kir6.1 Subunit in BV-2 Cell Lines

Microglia is the resident immune cells in the brain. Once stressed, they can be activated and produce some cytokines. However, the functions are determined by the activated state of microglia. As in the present study, BV-2 cells released lots of LDH to the medium since OGD 1 h and reoxygenation 24 h, but there was no significant change in cell viability (Figure 1A,B). After OGD 1.5 h and reoxygenation 24 h, there was a significant change in LDH release and cell viability (Figure 1A,B). These data suggested that OGD 1.5 h and reoxygenation 24 h could induce BV-2 cells death.

Under normoxia condition, BV-2 cells showed long fusiform in shape with slender processes. Exposure to OGD for 1 h and reoxygenation 24 h, the morphology of BV-2 cells became round with shorter processes, as shown in Figure 1C. Moreover, OGD-induced changes in protein expressions of K_{ATP} channel subunits (Figure 1D). The protein expressions of Kir6.1 subunit were downregulated by 50% after OGD 1 h and reoxygenation 24 h ($P < 0.05$), whereas SUR1 subunit was upregulated about 53% ($P < 0.01$). OGD did not alter the protein levels of SUR2A and SUR2B. Thus, these results indicated Kir6.1/K_{ATP} channels expressed in BV-2 cells might be involved in OGD-induced microglial activation.

Nicorandil Inhibits OGD-Induced Releases of Pro-Inflammatory Cytokines from BV-2 Cells

To identify the effects of K_{ATP} channels expressed in microglia on OGD-induced inflammation, we respectively treated BV-2 cells with K_{ATP} channel opener (Nicorandil, 10 μM) and blocker (PNU37883, 20 μM) 1 h prior to OGD. As shown in Figure 2A, nicorandil treatment prevented OGD-induced variance of cellular morphology and BV-2 cells remained ramified instead of being activated. However, PNU37883 failed to prevent BV-2 cells being activated. Furthermore, we detected inflammatory cytokines in the medium. After OGD 1 h and reoxygenation 24 h, pro-inflammatory factors tumor necrosis factor-alpha (TNF-α) (619.62 $\pm$ 85.16 pg/mL) (Figure 2B) and IL-1β (119.51 $\pm$ 5.05 pg/mL) (Figure 2C) produced from BV-2 cells were greatly enhanced ($P < 0.01$), compared with control group (TNF-α: 315.71 $\pm$ 27.41 pg/mL, IL-1β: 60.93 $\pm$ 4.42 pg/mL). Moreover, it could also induce significant downregulation of IL-10 ($P < 0.05$) (Figure 2D). However, pretreatment with nicorandil (10 μM) significantly reduced TNF-α (reduction by 34.53%) and IL-1β (reduction by 17.23%) production from BV-2 cells after OGD ($P < 0.05$). While pretreatment with PNU37883 (20 μM) failed to decrease inflammatory factors release after OGD. Both nicorandil and PNU37883 did not affect OGD-induced downregulation of IL-10. All these results suggested that opening K_{ATP} channels inhibited

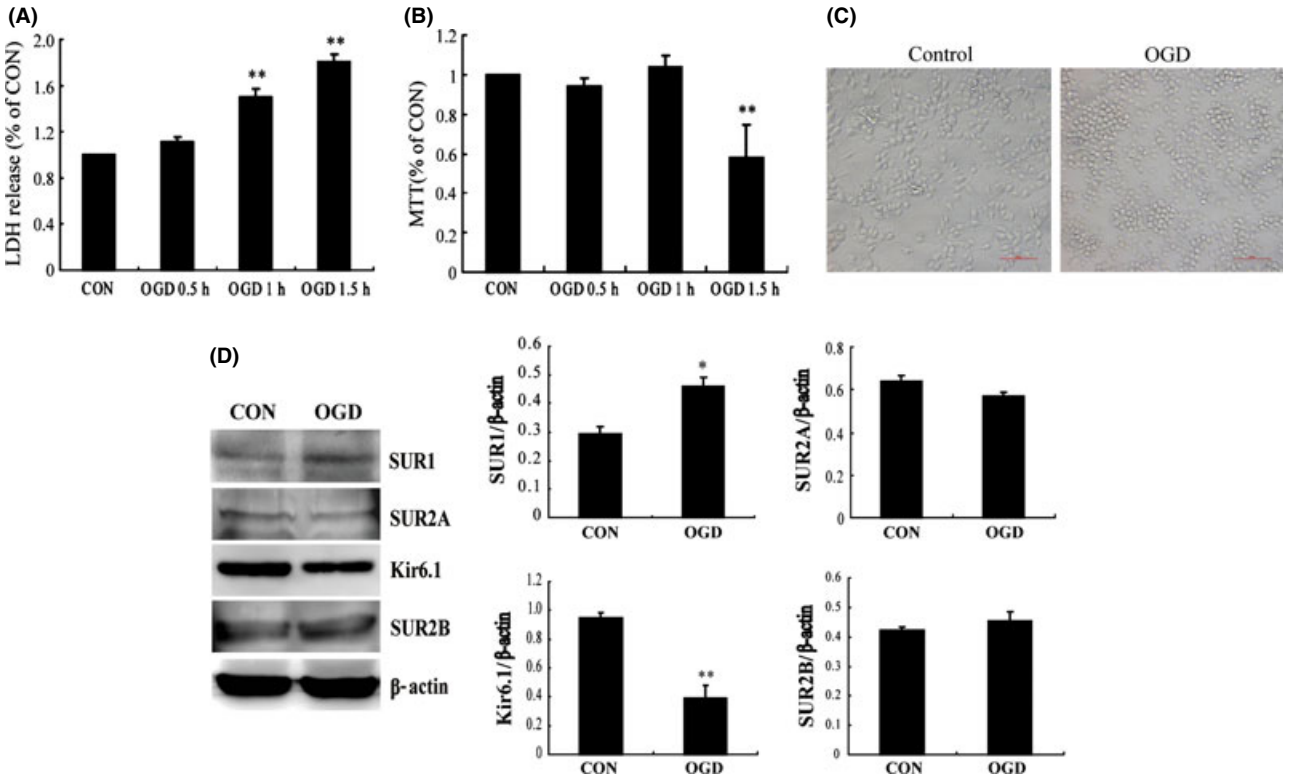

Figure 1 Oxygen–glucose deprivation (OGD) induces downregulation of Kir6.1 subunit in activated BV-2 cells. The timecourse of lactate dehydrogenase (LDH) release (**A**) and MTT activity (**B**) of BV-2 cells after OGD/reoxygenation. (**C**) The representative morphological alteration of BV-2 cells after OGD 1 h and reoxygenation 24 h. Scale bar: 40 μM. (**D**) Western blotting analyses and represented immunoblots of K_{ATP} channels subunits in BV-2 cells. *$P < 0.05$, **$P < 0.01$ versus control group. Results are shown as mean $\pm$ SEM of every three individual experiments.

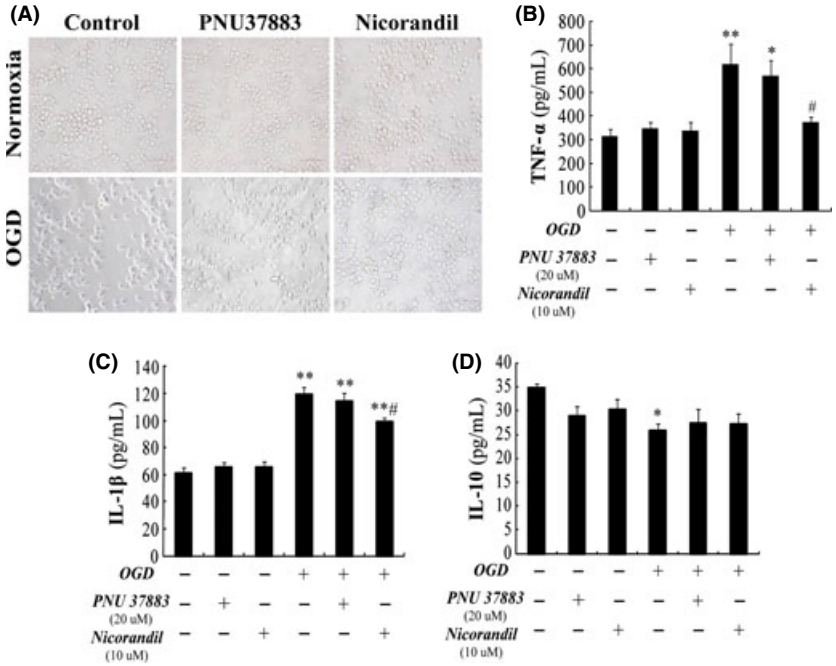

Figure 2 Opening K_{ATP} channels decreases production of pro-inflammatory cytokines from activated BV-2 cells. (**A**) The represented appearance of BV-2 cells under microscope. Scale bar: 40 μM. ELISA assays of tumor necrosis factor-alpha (TNF-α) (**B**), Interleukin-1beta (IL-1β) (**C**), and IL-10 (**D**) in the medium of BV-2 cells. *$P < 0.05$, **$P < 0.01$ versus lane 1; #$P < 0.05$ versus lane 4. Results are shown as mean ± SEM of every seven individual experiments.

pro-inflammatory cytokines release from microglia induced by OGD.

Nicorandil Downregulates TLR4 and Inhibits Phosphorylation of IKK

Because activation of microglial cells in response to OGD is characterized by upregulation of TLRs, which drives downstream signaling pathway to promote the expression of inflammatory genes and cytokines. We next investigated how this process initiated after OGD and how did K_{ATP} channels work. Our results showed that the expression of TLR4 (Figure 3A,B) and phosphorylation of IkappaB kinase (IKK) (Figure 3A,C) increased notably after OGD 1 h and reoxygenation 24 h ($P < 0.01$). However, treatment with nicorandil (10 μM) remarkably decreased the expression of TLR4 (reduction by 29.39%) and phosphorylated IKK (reduction by

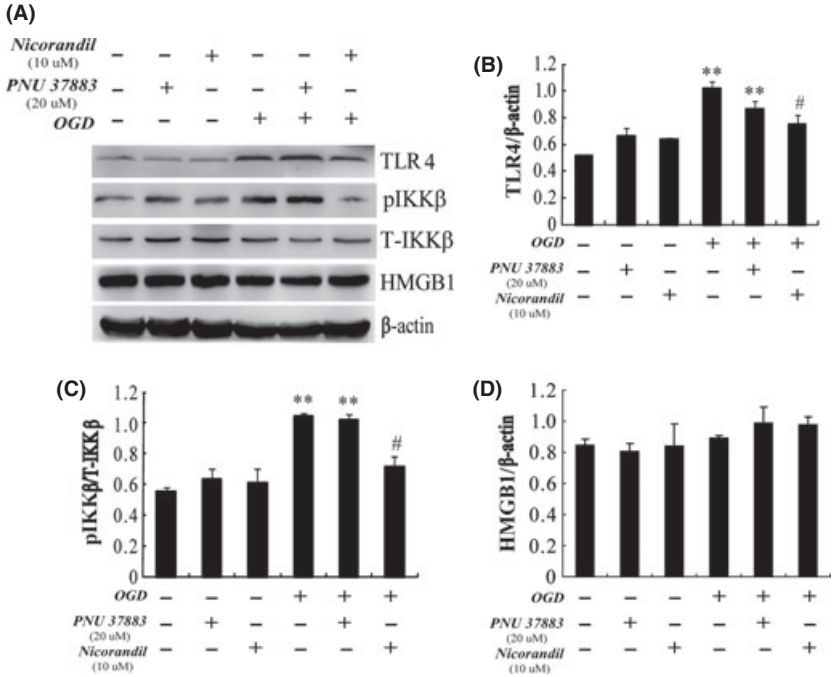

Figure 3 Opening K_{ATP} channels downregulates Toll-like receptor-4 (TLR4) and phosphorylated IkappaB kinase (IKK) complex in activated BV-2 cells. (**A**) Represented immunoblots of TLR4, IKKβ, and high mobility group box 1 (HMGB1). Western blotting analyses of TLR4 (**B**), pIKK (**C**), and HMGB1 (**D**) in BV-2 cells. **$P < 0.01$ versus lane 1; #$P < 0.05$ versus lane 4. Results are shown as mean ± SEM of every three individual experiments.

26.91%) ($P < 0.01$), but closing Kir6.1/K_{ATP} channels by PNU37883 (20 μM) could not produce this potency. These obtained results indicated that opening K_{ATP} channels inhibited TLR4/IKK signaling pathway and thereby prevented neuroinflammation induced by OGD. High mobility group box 1 (HMGB1) is a DNA-binding nuclear protein, which can be released into the medium during the state of stress. Released HMGB1 is a biomarker of necrosis. As shown in Figure 3D, expression of HMGB1 did not change after OGD 1 h and reoxygenation 24 h, and pretreatment with nicorandil or PNU37883. These results suggested that BV-2 cells were only activated when there was no cell death.

Nicorandil Inhibits the Activation of NLRP3 Inflammasome

As we know IL-1β is one of the most important pro-inflammatory mediators. Previous findings have shown that NLRP3 inflammasome is most significantly associated with maturation of IL-1β and involved in sterile inflammation. According to these findings, we further wanted to confirm whether activation of NLRP3 inflammasome was involved in OGD-induced production of IL-1β. We found that OGD could lead to upregulation of mature IL-1β (increase about 3-fold). Pretreatment with nicorandil not PNU37883 could reverse this phenomenon ($P < 0.05$) (Figure 4A, D). Furthermore, we examined expressions of NLRP3 and cleaved Caspase-1 by Western blotting (Figure 4A). Consistently, the expressions of NLRP3 (Figure 4B) and cleaved caspase-1 (Figure 4C) were both significantly increased after OGD ($P < 0.05$), but pretreatment with nicorandil (10 μM) suppressed the upregulation of NLRP3 and cleaved caspase-1 in response to OGD. However, the blocker PNU37883 was ineffective. These results suggested that opening the Kir6.1/K_{ATP} channels could inhibit inflammasome activation and ameliorated OGD-induced production of IL-1β in BV-2 cells, although its molecular mechanisms should need to be further investigated in future study.

Discussion

Post-ischemic inflammation is widely believed to exacerbate neuron death in cerebral ischemia and reoxygenation [17]. Microglia is the main resource of pro-inflammatory cytokines at the early stage of cerebral ischemia [18]. So, the regulation of microglial activation and microglia-mediating neuroinflammation is considered to be an urgent therapeutic strategy for stroke. Kir6.1-contained K_{ATP} channels are the main subtypes expressed in microglia [11,12]. In the present study, we found that OGD downregulated Kir6.1 subunit expression in BV-2 cell lines. Downregulation of Kir6.1 might be concerned with prompt energy exhaust or glucose deprivation induced K_{ATP} channels trafficking [13,19]. Moreover, it was reported that Kir6.1 gene knockout mice were susceptible to LPS stimulus, which lead to endotoxemia and death [15]. Our previous study has also clarified Kir6.1 knockdown aggravates microglal activation and inflammatory responses after cerebral ischemia injury [13]. In the present study, we found that microglia was activated after OGD 1 h and reoxygenation 24 h and released massive pro-inflammatory factors, such as TNF-a and IL-1β. Moreover, we found that OGD downregulated Kir6.1 subunit expression in BV-2 cells. Then, we pretreated BV-2 cells with the K_{ATP} channel opener (nicorandil) and blocker (PNU37883), respectively. The data showed that pretreatment with nicorandil could attenuate BV-2 cells activation and prevented the productions of TNF-a and IL-1β, but PNU37883 failed to inhibit inflammatory responses induced by OGD in BV-2 cells. These findings were consistent with other reports that opening K_{ATP} channels was favorable for sterile inflammation [11,12]. However, the molecular mechanisms remain unclear.

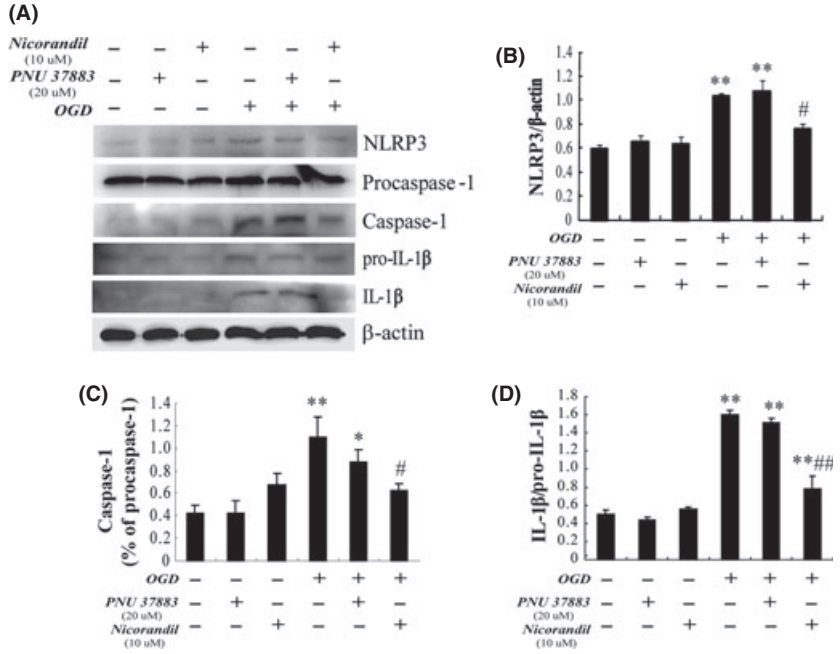

Figure 4 Opening K_{ATP} channels suppresses inflammasome activation and production of interleukin-1beta (IL-1β) induced by oxygen–glucose deprivation (OGD). (**A**) Represented immunoblots of NLRP3, cleaved Caspase-1, and IL-1β. Western blotting analyses of NLRP3 (**B**), cleaved Caspase-1 (**C**), and maturation of IL-1β (**D**). *$P < 0.05$, **$P < 0.01$ versus lane 1; #$P < 0.05$, ##$P < 0.01$ versus lane 4. Results are shown as mean ± SEM in every four individual experiments.

Recent evidence implicates the importance of TLR signaling in response to inflammatory injury [20,21]. TLR4 is an important member of transmembrane receptor family, which is consistently expressed in microglia and mediates innate immunity. The present study showed OGD 1 h and reoxygenation 24 h induced upregulation of TLR4 and phosphorylation of the IKK complex in BV-2 cells. It is reported that expression of TLR4 upregulated in microglia in response to hypoxia or ischemia; meanwhile, expression of phosphorylated IKK complex increased and promoted nuclear factor kappa B (NF-κB) translocation to the nucleus, all these lead to production of pro-inflammatory cytokines [20,22]. Consistently, TLR4 knockout mice showed significant smaller infarct size, improved behavioral outcomes and suppressed IKK complex phosphorylation, NF-κB activity and production of pro-inflammatory cytokines including TNF-a and IL-1β [23,24]. These suggested that OGD/reoxygenation (OGD/R)-induced production of pro-inflammatory cytokines associated with TLR4/IKK signaling pathway. Additionally, we found that OGD/reoxygenation could also induce upregulation of NLRP3 and cleaved caspase-1. NLRP3 expression depends on priming stimulus. After acute injury such as cerebral ischemia, there are many endogenous molecules, which can be served as priming stimulus to initiate NLRP3 expression. But its mechanisms remain poorly characterized. Recently, it has been confirmed that NLRP3 inflammasome can be activated by diverse molecules including extracellular ATP, Aβ and elevated plasma glucose [25–27]. The latest study reported that acute-phase protein serum amyloid A (SAA) acted as priming stimulus on glial cells resulting in inflammasome activation [28]. Additionally, extracellular PH or rodex also exerted great contribution to activating NLRP3 inflammasome [29,30]. However, which is the main contributor to the upregulation of NLRP3 and caspase-1 activation under OGD condition needs to be further studied. At least, our data indicated OGD/R-induced inflammatory responses via regulating TLR4/IKK signaling pathway and NLRP3 inflammasome activation. To explore the molecular mechanisms of inflammasome activation under OGD/R condition, its intervention deserves to be focused.

The accumulating evidence indicates that K_{ATP} channels correlate with glial inflammation. Besides, opening K_{ATP} channels could inhibit inflammatory cytokines transcription. Our present study showed that pretreatment with nicorandil significantly suppressed the expressions of TLR4, NLRP3, phosphorylation of IKK complex and cleaved caspase-1 induced by OGD, and thereby reduced overproduction of inflammatory factors, such as TNF-a and IL-1β. Nicorandil is a potassium channel opener and nitric oxide (NO) donor, which is clinically utilized as a nitrovasodilator and reduce smooth muscle contraction. There is increasing evidence suggests that nicorandil is involved in modulation of inflammation, but its molecular mechanisms remain unclear [31,32]. These data revealed that nicorandil could inhibit TLR4/IKK signaling pathway and inflammasome activation, which protected BV-2 cells against inflammatory responses after OGD/R injury. However, PNU37883 (Kir6.1 blocker) failed to do it. These suggest that Kir6.1-contained K_{ATP} channels might play an essential part in glial neuroinflammation.

In summary, our data revealed that opening Kir6.1-contained K_{ATP} channels expressed in microglia could inhibit inflammasome activation and TLR4 signal transduction and consequently prevented OGD/R-induced neuroinflammation. These findings suggest that opening K_{ATP} channels is favorable for post-ischemic inflammation, contributing to limiting microglial activation and overproduction of pro-inflammatory cytokines. However, the detailed molecular mechanisms involved in Kir6.1-contained K_{ATP} channels regulating inflammasome activation need to be clarified in future study.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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