Elevated intracranial pressure induces IL-1β and IL-18 overproduction via activation of the NLRP3 inflammasome in microglia of ischemic adult rats

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Abstract. Elevated intracranial pressure (ICP) is one of the most common complications following an ischemic stroke, and has implications for the clinical and neurological outcomes. The aim of the present study was to examine whether elevated ICP may increase IL-1β and IL-18 secretion by activating the NOD-like receptor protein 3 (NLRP3) inflammasome in microglia of ischemic adult rats. Sprague-Dawley rats that underwent middle cerebral artery occlusion were used for assessment of ICP. Reactive oxygen species (ROS) production was detected, and western blotting and immunofluorescence staining were used to determine the expression levels of caspase-1, gasdermin D-N domains (GSdMD-N), IL-1β and IL-18 in microglial cells. ICP levels were significantly increased, which was accompanied by ROS overproduction, in the brain tissue following ischemia-reperfusion (IR) injury in rats. Treatment with 10% hypertonic saline by intravenous injection significantly reduced the ICP and ROS levels of the rats. Furthermore, high pressure (20 mmHg) combined with oxygen-glucose deprivation (OGd) treatment resulted in increased ROS production in BV-2 microglial cells compared with those subjected to OGd treatment alone in vitro. Elevated pressure upregulated the expression of Caspase-1, GSdMD-N, IL-18 and IL-1β in IR-treated or OGd-treated microglia both in vivo and in vitro. More importantly, Caspase-1, GSdMD-N, IL-18 and IL-1β expression in microglia was significantly downregulated when elevated pressure was reduced or removed. These results suggested that elevated ICP-induced IL-1β and IL-18 overproduction via activation of the NLRP3 inflammasome by ischemia-activated microglia may augment neuroinflammation.

Introduction

Ischemic stroke is one of the most common causes of death worldwide (1,2). Cerebral edema and elevated intracranial pressure (ICP) are common complications following ischemic stroke (3,4). Elevated ICP is a key factor affecting the clinical and neurological outcomes of stroke (5-7); however, the underlying mechanisms remain unclear.

Inflammation in the central nervous system is an important cause of secondary brain injury that occurs following cerebral ischemia (8). The microglial cells are the resident immunocytes in the brain and are activated within the first few hours following cerebral infarction and release a large number of inflammatory cytokines (9-12). Recent studies have reported that intracellular NOD-like receptors, including NOD-like receptor protein 3 (NLRP3), are widely expressed in microglia (13-16). NLRP3 inflammasome serves a key role in initiating and amplifying the inflammation in the central nervous system (17-19). To exert its functions, the activation of the NLRP3 inflammasome is first required. As several studies have reported, the NLRP3 inflammasome can be triggered by reactive oxygen species (ROS) (20-23). The expression of IL-1β and IL-18 can be upregulated following activation of the NLRP3 inflammasome, which promotes inflammation in the central nervous system and eventually leads to the aggravation of brain injuries following ischemic stroke (24,25). Whether ICP can mediate NLRP3 inflammasome activation in ischemic microglia remains to be clarified.

In the present study, it was hypothesized that elevated ICP may aggravate nerve injury that occurs following cerebral ischemia. The possible underlying mechanism was determined to involve elevated ICP, which in-turn increases IL-1β and IL-18 secretion via activation of the NLRP3 inflammasome.
Materials and methods

Animals and treatment. Male adult Sprague-Dawley rats aged 3-4 months weighing 220-250 g were provided by the Institute of Laboratory Animal Science of Jinan University (Guangzhou, China). The rats were fed standard chow and water, and housed under standard experimental conditions (temperature, 20-25°C; humidity, 50-70%) with a 12-h light/dark cycle for a week. As few animals as possible were used in the experiments. A total of 160 rats were randomly divided into four groups (n=40 rats per group): i) Sham-operated group; ii) cerebral ischemia-reperfusion (IR) group (IR group); iii) cerebral IR + normal saline group (NS group); and iv) cerebral IR + 10% hypertonic saline group (HS group). Rats in the IR, NS and HS groups were subjected to middle cerebral artery occlusion (MCAO). Rats in the sham group were subjected to all the procedures without occlusion. The tail vein was cannulated for intravenous infusion of 10% HS or normal saline. After IR, the rats in the NS group and HS group were continuously administered NS (0.3 ml/h) and 10% HS (0.3 ml/h) by intravenous injection, respectively. All animals were observed closely for 24 h.

Rat model of cerebral ischemia. Before the surgical procedure, all rats were fasted with access to water overnight. Cerebral ischemia was induced by right-sided MCAO as described previously (26). The rats were anesthetized with pentobarbital sodium (30 mg/kg intraperitoneal injection) followed by a midline incision. The right common carotid artery, internal carotid artery and external carotid artery were carefully exposed. A head-end spherical nylon suture was inserted from the external carotid artery into the middle cerebral artery until resistance was felt. The suture remained in place for 2 h, after which it was withdrawn to allow reperfusion. The health and behavior of the rats were monitored every 2 h after surgery. The rats (n=23) who could not walk spontaneously and had a depressed level of consciousness, were excluded from the study. The rats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneal injection) and euthanized with 0.9% sodium chloride intravenous perfusion and arterial exsanguination. The Research Ethics Committee of Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences approved all animal procedure protocols [approval no. GDREC2012106A(R1); Guangzhou, China].

Measurement of ICP. The ICP was measured 0, 2, 4, 8, 12, 16, 20 and 24 h after surgery (n=8 for each group). To evaluate the ICP, a midline incision over the vertex was performed following anesthesia, and then a hole caudal to the coronal suture was drilled, 4 mm from the midline. The dura was punctured and a microsensor for ICP was inserted intracranially (3). An ICP monitor (Integra CAMO2; Integra LifeSciences) was used to measure the ICP.

Measurement of ROS levels in brain tissue. The ROS levels in brain tissues were evaluated using a ROS ELISA kit (cat. no. DG21175D-96; DG Biotech) 24 h after IR. Briefly, samples and standards (50 µl/well) were added to the plate wells coated with HRP-conjugated antibodies, which were used to capture ROS. The plates were incubated for 1 h at 37°C. After washing completely, substrate A (50 µl/well) and substrate B (50 µl/well) were added to incubate the plate in the dark for 15 min at 37°C. Then, the stop buffer was added, and the optical density was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of ROS in the samples were then determined by comparing the optical density of the samples to the standard curve.

ROS measurement in microglia. The ROS production in the BV-2 microglial cells was evaluated using a ROS assay kit (cat. no. BB-4705-2; BestBio), according to the manufacturer's protocol. Briefly, DCFH-DA was diluted with DMEM high glucose without FBS (1:1,500). The coverslips with adherent BV-2 microglial cells were cultured in DMEM high glucose supplemented with 10% FBS. Following treatment, the medium was changed to diluted DCFH-DA (2 µl/well). Then, the plates were incubated for 20 min at 37°C and 5% CO2. The coverslips were washed with DMEM high glucose without FBS. Finally, the coverslips were mounted using a fluorescent mounting medium and visualized under a fluorescence microscope (Olympus DP73 Microscope; Olympus Corporation).

Western blotting analysis. Total proteins from the peri-infarcted cerebral cortex and BV-2 microglial cells (n=4 per group) were extracted using a Total Protein Extraction kit (cat. no. BB-3101-100T; BestBio) as described previously (26). Protein concentration was determined using a Pierce™ BCA Protein assay kit (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein from each sample (40 µg per lane) were separated via 10% SDS-PAGE, and then transferred to PVDF membranes, which were blocked with 5% non-fat milk for 1 h at room temperature. Subsequently, the following primary antibodies were added to incubate the membranes overnight at 4°C: Caspase-1 (1:1,000; cat. no. 24232S; Cell Signaling Technology, Inc.), IL-1β (1:1,000; cat. no. 12703S; Cell Signaling Technology, Inc.), IL-18 (1:1,000; cat. no. ab207323; Abcam) and gasdermin D-N domains (GSDMD-N; 1:1,000; cat. no. 36425S; Cell Signaling Technology, Inc.). The membranes were washed the following day, and the HRP-conjugated goat anti-rabbit antibody (1:2,000; cat. no. 7074S; Cell Signaling Technology, Inc.) was
added and the membrane was incubated for 2 h at 4°C. The immunoblots were visualized using a chemiluminescence kit (Bioworld Technology, Inc.), and detected using an imaging densitometer (ImageQuant™ LAS 500; Cytiva). The relative density was semi-quantified using FluorChem 8900 software (version 4.0.1; ProteinSimple). β-actin was used as the loading control.

Double immunofluorescence labeling. After 24 h of reperfusion, the rats were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneal injection) and transcardially perfused with saline and 4% paraformaldehyde sequentially. The brains were harvested and post-fixed in 4% paraformaldehyde for 24 h at 4°C. These tissue samples were then dehydrated in a graded series of sucrose solutions, embedded in optimal cutting temperature compound and cut into 10-µm thick sections.

In vitro, the coverslips with adherent BV-2 microglial cells were fixed with 4% paraformaldehyde for 20 min at room temperature 24 h after treatment. The sections/cover slips were blocked in 5% normal donkey serum (cat. no. ab7475; Abcam) for 0.5 h at room temperature. Subsequently, they were incubated with the following primary antibodies overnight at 4°C: Caspase-1 (1:100; cat. no. 24232S; Cell Signaling Technology, Inc.), IL-1β (1:100; cat. no. 12703S; Cell Signaling Technology, Inc.), IL-18 (1:100; cat. no. ab207323; Abcam), and Iba1 (1:100; cat. no. ab15690; Abcam). The sections/cover slips were washed the following day, and the secondary antibodies, Alexa Fluor® 549 goat anti-rabbit IgG (H+L) (1:100; cat. no. A11057; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa Fluor® 488 Goat anti-mouse IgG (1:100; Invitrogen; cat. no. A11029; Thermo Fisher Scientific, Inc.) were added to the sections and incubated for 1 h at room temperature. Finally, the sections were mounted using a fluorescent mounting medium with DAPI (Sigma-Aldrich; Merck KGaA) and visualized using a fluorescence microscope.

Statistical analysis. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc.). All values are expressed as the mean ± standard error of the mean. Repeated measures ANOVA was used to analyze the repeated measurement data. A one-way ANOVA was used to analyze the data of three or four-group univariate-factor measurements. Following ANOVA, multiple comparisons were performed using Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

ICP levels following MCAO. The ICP levels in the IR group and the IR + NS group were significantly higher than the sham group (P<0.01). There were no significant differences between the IR group and the IR + NS group (P>0.05). The ICP levels in the IR + HS group were significantly lower compared with the IR group (P<0.01; Fig. 1A-C; Table I).

Elevated ICP promotes ROS overproduction. The ROS levels in the IR group and the IR + NS group were significantly higher compared with the sham group (P<0.01). There was no significant difference between the IR group and the IR + NS group (P>0.05). The ROS levels in the IR + HS group were significantly lower compared with the IR group when ICP levels were reduced by HS (P<0.01; Fig. 2A). In vitro, increased ROS immunofluorescence was observed in the
Elevated ICP activates NLRP3 inflammasome.

Compared with the OGD + 20 mmHg group, ROS fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 2B).

**Elevated ICP promotes Caspase-1 activation.** In vivo, the protein expression levels of Caspase-1 were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There was no significant difference between the IR group and the IR + NS group (P>0.05). The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS (P<0.01; Fig. 3A and B). Double immunofluorescence staining was used to examine Caspase-1 expression in the microglia of the peri-infarcted brain tissue. Increased Caspase-1 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, Caspase-1 fluorescence was noticeably attenuated (Fig. 3C).

In vitro, the protein expression levels of Caspase-1 were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment.

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**Table I. ICP levels 0, 2, 4, 8, 12, 16, 20 and 24 h after ischemia-reperfusion in vivo.**

| Time Group, h | Sham, mmHg | IR, mmHg | IR + NS, mmHg | IR + HS, mmHg |
|---------------|------------|----------|---------------|---------------|
| 0             | 7.89±0.72  | 8.26±0.74 | 8.01±0.69     | 7.98±0.82     |
| 2             | 8.25±1.10  | 8.23±0.68 | 8.46±0.89     | 9.34±0.77     |
| 4             | 7.75±1.02  | 11.56±1.26 | 12.48±0.75   | 10.63±0.82    |
| 8             | 7.83±0.86  | 14.31±1.01 | 14.60±1.03   | 11.91±1.02    |
| 12            | 7.31±0.72  | 17.39±1.12 | 17.04±0.87   | 12.75±1.10    |
| 16            | 7.49±0.85  | 20.55±1.30 | 19.44±0.84   | 13.56±0.93    |
| 20            | 7.63±0.65  | 21.05±1.00 | 21.43±1.03   | 13.89±1.00    |
| 24            | 7.94±1.09  | 22.35±0.84 | 23.33±0.98   | 13.58±1.00    |

n=8 per group. *P<0.01 vs. sham group; **P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group.
treatment (P<0.01; Fig. 4A and B). Double immunofluorescence staining was used to examine Caspase-1 expression in the BV-2 microglial cells. Increased Caspase-1 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 4C).

**Elevated ICP increases IL-1β expression.** The protein expression levels of IL-1β in vivo were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There were no significant differences between the IR and the IR + NS group (P>0.05). The IL-1β expression levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS (P<0.01; Fig. 5A and B). Double immunofluorescence staining was used to examine IL-1β expression in the microglia of the peri-infarcted brain tissue. Increased IL-1β immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. When ICP levels were reduced by HS, IL-1β fluorescence was notably attenuated (Fig. 5C).
In vitro, the protein expression levels of IL-1β were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 6A and B). Double immunofluorescence staining was used to examine IL-1β expression in the BV-2 microglial cells. Enhanced IL-1β immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, the fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 6C).

Elevated ICP increases IL-18 expression. The protein expression levels of IL-18 in vivo were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There were no significant differences between the IR group and the IR + NS group (P>0.05). IL-18 expression levels in the IR + HS group were significantly lower when ICP levels were reduced by HS compared with the IR group.
(P<0.01; Fig. 7A and B). Double immunofluorescence staining was used to examine IL-18 expression in the microglia of the peri-infarcted brain tissue. Enhanced IL-18 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. In the IR group, IL-18 fluorescence was notably decreased. Scale bar, 20 µm. n=4 per group. **P<0.01 vs. sham group; ***P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.

In vitro, the protein expression levels of IL-18 were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 8A and B). Double immunofluorescence staining was used to examine IL-18 expression in the BV-2 microglial cells. Enhanced IL-18 immunofluorescence was observed in the OGD + 20 mmHg group compared with the control group. Compared with the OGD + 20 mmHg group, fluorescence was notably reduced in the OGD group without high-pressure treatment (Fig. 8C).
Elevated ICP increases GSDMD-N expression. The protein expression levels of GSDMD-N in vivo were significantly increased in the IR group and the IR + NS group compared with the sham group (P<0.01). There were no significant differences between the IR and the IR + NS group (P>0.05). The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS (P<0.01; Fig. 9A and B).

In vitro, the protein expression levels of GSDMD-N were significantly increased in the OGD + 20 mmHg group compared with the control group (P<0.01). Compared with the OGD + 20 mmHg group, the expression levels were significantly reduced in the OGD group without high-pressure treatment (P<0.01; Fig. 9C and D).

Discussion

In the present study, it was shown that elevated ICP promoted NLRP3 inflammasome activation in microglia of ischemic adult rats. This was evident based on the increased expression levels of Caspase-1, GSDMD-N, IL-18 and IL-1β in the ischemia-activated microglial cells.
ICP management is an essential means of preventing secondary injury in the central nervous system following ischemic stroke (5-7). Osmotherapy has been used as a foundation for managing elevated ICP levels induced by cerebral edema (5,6). Commonly used osmotic agents include HS and mannitol (28,29). In the present study, to determine the effects of HS on ICP following ischemic stroke, the ICP of the rats were examined 0, 2, 4, 8, 12, 16, 20 and 24 h after MCAO. The ICP levels were significantly increased following cerebral IR. Following 10% HS treatment by intravenous injection, the ICP of the rats after MCAO was significantly reduced. This was consistent with the effects of HS on traumatic brain injury (30) and subarachnoid hemorrhage (31).

The NLRP3 inflammasome can be triggered by ROS (20-23). It has been reported that ROS levels are increased during high altitude exposure in lowlanders, which induced passive hypobaric hypoxia; optic nerve sheath diameter (ONSd), which is an indirect measurement of ICP, is concurrently increased. However, regression analysis did not infer a causal relationship between oxidative stress biomarkers and changes in ONSd (32). In the present study, the ROS levels of the brain tissue were increased when ICP was increased.

Figure 7. Elevated ICP increases IL-18 expression in microglia following IR in vivo. (A) Immunoreactive bands of IL-18 (22 kDa) and β-actin (43 kDa). (B) Protein expression levels of IL-18 were significantly increased in the IR group and the IR + NS group compared with the sham group. There was no significant difference between the IR group and the IR + NS group. The levels in the IR + HS group were significantly lower than the IR group when ICP levels were reduced by HS. (C) Immunofluorescence images showing the expression of (C-a, C-e, C-i and C-m) Iba1+ microglia (green), (C-b, C-f, C-j and C-n) IL-18 (red), (C-c, C-g, C-k and C-o) the co-localization of IL-18 and microglia, and (C-d, C-h, C-l and C-p) high amplification images of the microglial cells in peri-ischemic cortex. Increased IL-18 immunofluorescence was observed in the IR group and the IR + NS group compared with the sham group. In the IR + HS group, IL-18 fluorescence was notably reduced. Scale bar, 20 µm. n=4 per group. **P<0.01 vs. sham group; ##P<0.01 vs. IR group. ICP, intracranial pressure; sham, sham-operated; IR, ischemia-reperfusion; NS, normal saline; HS, hypertonic saline group; ns, non-significant.
following cerebral IR. Additionally, ROS overproduction was inhibited by reduced ICP. Furthermore, high pressure (20 mmHg) combined with OGD treatment increased ROS production in the BV-2 microglial cells compared with those subjected to OGD treatment alone in vitro. These results suggested that elevated ICP can enhance oxidative stress. To determine whether elevated ICP activated the NLRP3 inflammasome via ROS overproduction in ischemia-activated microglia, expression of Caspase-1, GSDMD-N, IL-18 and IL-1β in the microglia were determined both in vivo and in vitro. It was shown that elevated pressure upregulated the expression of Caspase-1, GSDMD-N, IL-18 and IL-1β in IR- or OGD-treated microglia both in vivo and in vitro. More importantly, Caspase-1, GSDMD-N, IL-18 and IL-1β expression in microglia was significantly downregulated when the elevated pressure was reduced or removed. These results suggested that elevated ICP increased NLRP3 inflammasome activation in ischemia-activated microglial cells via induction of ROS overproduction.

However, there are limitations to the present study. Firstly, it has been reported that activated microglia secrete pro-inflammatory cytokines, including TNF-α, IL-18 and IL-1β (33-35). However, the lack of investigation of other cytokines (such as TNF-α) in the present study, is a limitation
and an area for future research. Secondly, the present study did not determine the upstream mechanism by which elevated ICP promotes ROS overproduction in ischemic stroke. It has been found that ischemia damages mitochondria and induces ROS production (36,37). Mitophagy can eliminate damaged mitochondria, reduce ROS production and then alleviate NLRP3 inflammasome activation (38). Elevated ICP may promote ROS overproduction by inhibiting mitophagy. It has been reported that the NLRP3 inflammasome is expressed in astrocytes (39). However, the lack of determination of NLRP3 inflammasome expression in astrocytes in the present study is another limitation of the present study. Finally, all animals were observed closely for 24 h after IR in the present study. However, the lack of determination of factors after various time points is another limitation.

In summary, elevated ICP was found to upregulate the expression of Caspase-1, GSDMD-N, IL-18 and IL-1β in ischemic microglia, which was significantly downregulated when ICP was reduced. Thus, elevated ICP-induced Caspase-1, GSDMD-N, IL-18 and IL-1β overproduction in the microglia may be a potential target for mitigating neuroinflammation following an ischemic stroke.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ conceived the project and designed the experiments. HD carried out the assessment of ICP and ROS evaluation. YL established the rat model of MCAO. MW carried out BV-2 microglial cell cultures and treatment. XL and YH performed western blotting and immunofluorescence staining. HD conducted the statistical analysis. HD and YL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Research Ethics Committee of Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences approved all animal procedure protocols [approval no. GDREC2012106A(R1); Guangzhou, China].
Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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