

Hypercapnia intensifies cerebral hypoxia via increasing cerebral oxygen extraction ratio: implication in neuroinflammation in hypoxemic adult rats

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Research

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Abstract

Background: Hypoxemia is a typical symptom of acute respiratory distress syndrome (ARDS). Ventilations are always needed for correcting hypoxemia. To avoid pulmonary morbidity, the low tidal volume ventilation is often applied. The ventilation strategy will certainly result in hypercapnia. Our previous study found that hypercapnia increase neuronal death and aggravate the cognitive function of hypoxic adult rats. However, the underlying mechanism has remained unclear. This study aimed to explore whether hypercapnia would induce neuroinflammation through increasing cerebral oxygen extraction ratio in adult rats with hypoxemia.

Methods: Cerebral oxygen extraction ratio (CERO₂), partial pressure of brain tissue oxygen (PbtO₂), and reactive oxygen species (ROS) production in brain tissue in a rat model of hypercapnia/hypoxemia were evaluated. Along with this, the oxygen consumption rate (OCR) and ROS production of BV-2 microglial cells were evaluated after 15% CO₂/0.2% O₂ treatment. The protein expression level of caspase-1 and IL-1 β in microglia cells were detected before and after application of a ROS scavenger in vivo and in vitro.

Results: PbtO₂ level was elevated by hypercapnia in the hypoxemic rats in the first 1.5 h, but it was significantly decreased 2 h after ventilation. This was further evident by the increased levels of CERO₂ at 3 h after ventilation. Besides, a high concentration of CO₂ treatment could increase the levels of OCR in hypoxic BV-2 microglial cells in vitro. Hypercapnia markedly increased the production of ROS and the expression of caspase-1 and IL-1 β in hypoxia-activated microglia both in vivo and in vitro. Pharmacological scavenging ROS inhibited the NLRP3 inflammasome activation and expression of IL-1 β .

Conclusion: Hypercapnia-intensified cerebral hypoxia via increasing cerebral oxygen extraction ratio may induce ROS overproduction, activate the NLRP3 inflammasome and enhance IL-1 β release in hypoxia-activated microglia.

Background

Acute respiratory distress syndrome (ARDS) is a common critical disease in intensive care units (ICUs) [1,2]. Persistent hypoxemia is a typical symptom of ARDS. Ventilations are always needed to correct hypoxemia[3,4] To avoid or reduce pulmonary morbidity, the low tidal volume ventilation is often applied[5,6]. The ventilation strategy will certainly result in hypercapnia, which is called "permissive hypercapnia"[7,8]. Our previous study found that increase neuronal death and aggravate the cognitive function of hypoxic adult rats[9]. However, the underlying mechanism has remained unclear.

The NLRP3 inflammasome is widely expressed in microglia, which plays an important role in initiating neuroinflammation[10–12]. To exert its functions, the NLRP3 inflammasome requires to be activated. The NLRP3 inflammasome can be triggered by an array of stimuli, including ATP, potassium efflux, heme, urate, and reactive oxygen species (ROS)[13–17]. Activation of the NLRP3 inflammasome can upregulate the expression of caspase-1 and then cleavage pro-interleukin-1 beta (pro-IL-1 β) to IL-1 β . IL-1 β can promote the central nervous system cascade inflammatory response and lead to aggravation of neuronal

injury[18,19]. When the levels of oxygen fall, hypoxia would inhibit the respiratory chain and induce ROS production[20–22]. It remains to be ascertained whether hypercapnia would activate the NLRP3 inflammasome through increasing ROS overproduction in hypoxia-activated microglia.

Neurons and neuronal functions are highly sensitive to hypoxia. Under hypoxic conditions, the oxidative metabolism rate of the brain would decrease to prevent the partial pressure of brain tissue oxygen (P_{btO_2}) from dropping to a very low level[23,24]. However, it is still unknown whether hypercapnia would exert any effects on oxidative metabolism of the hypoxic brain.

In the present study, we hypothesized that hypercapnia may induce the NLRP3 inflammasome activation in microglia via intensifying cerebral hypoxia in adult rats with hypoxemia. It was surmised that hypercapnia might exert its effect through increasing $CERO_2$, decreasing P_{btO_2} , and inducing ROS production, which ultimately would activate the NLRP3 inflammasome in microglia and enhance the release of IL-1 β .

Methods

Animals and experimental groups

Male adult Sprague-Dawley (SD) rats (n = 128) aged about 3 months and weighing approximately 220 to 250 g were randomly divided into five groups: sham-operated group (abbreviated Sham group), Hypercapnia group, Hypoxemia group, Hypercapnia + Hypoxemia group (abbreviated HH group), Hypercapnia + Hypoxemia + N-Acetyl-L-cysteine group (abbreviated HH + NAC group).

The rat model of hypercapnia/hypoxemia

All rats were fasted with access to water overnight before the experiments. The rat model of hypercapnia/hypoxemia was established as described in our previous study[9]. Briefly, the rats were anesthetized with pentobarbital sodium followed by **mechanical ventilation**. The tidal volume (9ml/kg body weight), respiratory rate (45 breaths/min), and inspiratory to expiratory ratio (1:1) were fixed. Sham group was exposed to the air. Hypercapnia group was exposed to 5% CO_2 to maintain pH of arterial blood at 7.20 - 7.25. Hypoxemia group was exposed to 16% O_2 to maintain partial pressure of artery blood oxygen (PaO_2) at 55 - 60 mmHg. HH group was exposed to 16% O_2 mixing with 5% CO_2 to maintain PaO_2 at 55 - 60 mmHg and pH at 7.20 - 7.25. Rats in the HH + NAC group were treated with an intraperitoneal injection of 150 mg/kg NAC (MedChemExpress, Monmouth, NJ, USA; cat. no. HY-B0215) for 30 min before being exposed to 5% CO_2 and 16% O_2 . The rats, which were used for Western blotting analysis and immunofluorescence staining, were not performed invasive manipulation except ventilation.

Measurement of cerebral oxygen extraction ratio ($CERO_2$)

The right femoral artery and the right jugular vein was cannulated upstream. The blood samples were collected at 3 h after ventilation. The hemoglobin concentration (Hb), saturation of artery blood oxygen

(SaO₂), PaO₂, saturation of jugular vein blood oxygen (SjVO₂), and pressure of jugular vein blood oxygen (PjVO₂) were measured using a blood gas/electrolyte analyzer (Model 5700, IL, San Diego, CA, USA). The content of artery blood oxygen (CaO₂), content of jugular vein blood oxygen (CjVO₂), and CERO₂ were calculated using the following formulas:

[Due to technical limitations, please see the supplementary files section to access the formulas.]

Measurement of the partial pressure of brain tissue oxygen (PbtO₂)

The levels of PbtO₂ were measured at 0.5, 1, 1.5, 2, 2.5, and 3 h after ventilation. To evaluate the PbtO₂, a midline incision over vertex was performed after anesthesia. After this, a hole was drilled caudal to the coronal suture, 4 mm from the midline. The dura was punctured and a microsensor for PbtO₂ was inserted into brain tissue[24]. A monitor (Integra CAMO2, Integra LifeSciences Limited, County Offaly, Ireland) was used to measure the PbtO₂.

ROS evaluation of brain tissue

The ROS of brain tissue was evaluated using a ROS ELISA kit (Dogesce, Beijing, China; cat. no. DG21175D) at 3 h after ventilation following the manufacturers' instructions. Briefly, samples and standards (50 µl/well) were added to the plate wells coated by antibodies labeled with HRP, which were used to capture ROS. The plate was 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 a dark place for 15 min at 37 °C. Then the stop buffer was added, and the optical density (OD) 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

BV-2 microglial cell cultures and treatment

BV-2 microglial cells were purchased from CHI Scientific (cat. no. 7-1502), and were cultured and treated as described in our previous study[9]. Briefly, the cells were cultured with DMEM high glucose (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA; cat. no. 8117121) supplemented with 10% FBS (Invitrogen Life Technologies Corporation; Carlsbad, CA, USA; cat. no. 42F0374K). The microglial cells were randomly divided into five groups: Control group, high concentration of carbon dioxide group (abbreviated HC group), Hypoxia group, Hypoxia + HC group, and Hypoxia + HC + NAC group. Control group was exposed to 5% CO₂ + 20% O₂. HC group was exposed to 15% CO₂ + 20% O₂ to maintain pH of the supernatant at 7.20 - 7.25. Hypoxia group was exposed to 5% CO₂ + 0.2% O₂ to maintain partial pressure of oxygen (PO₂) of the supernatant at 55 - 60 mmHg. Hypoxia + HC group was exposed to 15% CO₂ + 0.2% O₂ to maintain PO₂ at 55 - 60 mmHg and pH at 7.20 - 7.25. The cells in the Hypoxia + HC + NAC group were treated with 2 mM NAC (MedChemExpress, Monmouth, NJ, USA; cat. no. HY-B0215) for 30 min before being exposed to 15% CO₂ + 0.2% O₂.

Oxygen consumption rate (OCR) evaluation of BV-2 microglial cells

The OCR was evaluated using a cellulate OCR Assay Kit (BestBio, Shanghai, China; cat. no. BB-48211) after treatment with 0.2% O₂ and 15% CO₂ for 0 h, 6 h, 12 h, and 24 h. The intervention time and testing time were chosen, when the levels of OCR peaked (In this study, 12 h was chosen as the intervention time, and 18 min was chosen as the testing time). The OCR was measured following the manufacturers' instructions. Briefly, BV-2 microglial cells were seeded in 96-well plates (5000 cells/well) and cultured in DMEM high glucose supplemented with 10% FBS. When the cells spread to 80% of the bottom of the well, they were treated with different concentrations of CO₂ and O₂. After the treatment, the medium was changed to DMEM high glucose without FBS, and then fluorescent probes (10 µl/well) were added sequentially. Finally, oxygen mounting medium was added (2 drops/well). The OCR levels were examined every three minutes until half an hour with a fluorescent microplate reader (Model 9260, IL-COR[®] inc, LINCOLN, NE, USA). The excited and emitted wavelength were 485/20 nm and 590/35 nm, respectively.

ROS measurement in microglia

The ROS production in BV-2 microglial cells was evaluated using a ROS assay kit (BestBio, Shanghai, China; cat. no. BB-4705-2) following the manufacturers' instructions. Briefly, DCFH-DA was diluted with DMEM high glucose without FBS (1: 1500). The coverslips with adherent BV-2 microglial cells were cultured with DMEM high glucose supplemented with 10% FBS. After the treatment, the medium was changed to diluted DCFH-DA (2 ml/well). Then the plates were incubated for 20 min at 37 °C, 5% CO₂. The coverslips were washed with DMEM high glucose without FBS. Finally, the coverslips were mounted by a fluorescent mounting medium and detected using a fluorescence microscope (Olympus DP73 Microscope, Olympus, Tokyo, Japan).

Western blotting analysis.

Total proteins from the hippocampus tissue and BV-2 microglial cells (n = 4 for each group) were extracted using a Total Protein Extraction Kit (BestBio, Shanghai, China; cat. no. BB-3101-100T). Protein concentrations were determined using a BCA Protein Assay Kit (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA; cat. no. 23227). Equal amounts of protein from each sample were separated in a 15% SDS-PAGE gel and transferred to PVDF membranes, which were blocked with 5% non-fat milk for 1 h at room temperature. After this, the following primary antibodies were added to incubate the membranes overnight at 4 °C: caspase-1 (1: 1000, Abcam, Cambridge, MA, USA; cat. no. ab1872) and IL-1β (1: 1000, Abcam, Cambridge, MA, USA; Cat. No. ab9722). The membranes were washed on the following day, and HRP-labeled goat anti-rabbit antibody (1: 3000; Cell Signaling Technology; cat. no. 7074S) was added to incubate the membranes for 2 h at 4 °C. The immunoblots were visualized using a chemiluminescence kit (Bioworld Technology, St. Louis Park, MN, USA; cat. no. AC36131), and detected by an imaging densitometer (ImageQuant LAS 500, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The relative density was quantified using FluorChem 8900 software (version 4.0.1, Alpha Innotech Corporation, San Leandro CA, USA). β-actin was used as the control.

Double immunofluorescence labeling

In vivo, the rats were anesthetized with pentobarbital sodium and transcardially perfused with normal saline and 4% paraformaldehyde at 3 h after ventilation. The brains were harvested and post-fixed in 4% paraformaldehyde. This tissue was then dehydrated in graded sucrose and cut into sections of 10 μm thickness. The sections were blocked in 5% normal donkey serum for 0.5 h at room temperature. *In vitro*, the coverslips with adherent BV-2 microglial cells were fixed with 4% paraformaldehyde at 24 h after treatment. The coverslips were blocked in 5% normal donkey serum for 0.5 h at room temperature sequentially.

After that, the following primary antibodies were added to incubate the sections/coverslips overnight at 4 °C: caspase-1 (1: 100; Abcam, Cambridge, MA, USA; cat. no. ab1872), IL-1 β (1: 100; Abcam, Cambridge, MA, USA; cat. no. ab9722), and Iba1 (1: 100; Abcam, Cambridge, MA, USA; cat. no. ab15690). The sections/coverslips were washed on the following day, and the secondary antibodies Alexa Fluor® 549 Goat Anti-Rabbit IgG (H + L) (1:100; Invitrogen Life Technologies, Carlsbad, CA, USA; cat. no. ATRJN1301) and Alexa Fluor® 488 Goat anti-mouse IgG (1:100; Invitrogen Life Technologies, Carlsbad, CA, USA; cat. no. ATRMR2301) were added to incubate the sections/coverslips for 1 h at room temperature. Finally, the sections/coverslips were mounted by the fluorescent mounting medium with DAPI (Sigma, St. Louis, MO, USA; cat. no. SLBW4468) and detected using a fluorescence microscope (Olympus DP73 Microscope, Olympus, Tokyo, Japan).

Statistical analysis

The statistical analysis was performed by the SPSS19.0 statistical (IBM, New York, USA). All values are expressed as mean \pm standard deviation. Repeated measures one-way analysis of variance (ANOVA) was used to analyze the repeated measurement data. Factorial ANOVA was for the interaction effects. When an interaction was examined, simple effects analyses were evaluated. Differences were considered statistically significant if the *P* value < 0.05.

Results

Hypercapnia increased cerebral oxygen extraction rate (CERO₂) in hypoxemic rats

Significant interaction effects were observed between hypercapnia treatment and hypoxia treatment (*P* < 0.01) (Fig. 1a). Simple effects analyses found an increased CERO₂ in Hypoxemia group (*P* < 0.05), but not in Hypercapnia group (*P* > 0.05) compared with Sham group. HH group had the highest CERO₂ levels as compared with Hypoxemia group (*P* < 0.01) and Hypercapnia group (*P* < 0.01) (Fig. 1b).

Hypercapnia decreased partial pressure of brain tissue oxygen (PbtO₂) in hypoxemic rats

Compared with the Sham group, decreased PbtO₂ levels were found in Hypoxemia group (0.5 h - 3 h: *P* < 0.01), but not in Hypercapnia group (0.5 h - 3 h: *P* > 0.05) (Fig. 2b, c). HH group had higher PbtO₂ levels as

compared with Hypoxemia group in the first 1.5 hours (0.5 h: $P < 0.01$; 1 h: $P < 0.01$; 1.5 h: $P < 0.05$) (Fig. 2b). On the contrary, HH group had lower PbtO₂ levels as compared with Hypoxemia group after 2 hours (2.5 h: $P < 0.05$; 3 h: $P < 0.05$) (Fig. 2c).

Hypercapnia induced overproduction of ROS in hypoxemic hippocampus

Significant interaction effects were observed between hypercapnia treatment and hypoxia treatment ($P < 0.05$) (Fig. 3a). Simple effects analyses found increased levels of ROS in Hypoxemia group ($P < 0.01$), but not in Hypercapnia group ($P > 0.05$) compared with Sham group. HH group had the highest ROS levels as compared with Hypoxemia group ($P < 0.01$) and Hypercapnia group ($P < 0.01$) (Fig. 3b).

Hypercapnia enhanced NLRP3 inflammasome activation via inducing ROS overproduction in microglia in the hypoxic hippocampus

Significant interaction effects were observed between hypercapnia treatment and hypoxia treatment ($P < 0.01$) (Fig. 4b). Simple effects analyses found increased protein expression levels of caspase-1 in Hypoxemia group ($P < 0.01$), but not in Hypercapnia group ($P > 0.05$) compared with Sham group. HH group had the highest expression levels of caspase-1 as compared with Hypoxemia group ($P < 0.01$) and Hypercapnia group ($P < 0.01$) (Fig. 4c). Additionally, the protein expression of caspase-1 was significantly suppressed with NAC pretreatment (150 mg/kg) in rats ($P < 0.01$) (Fig. 4c). Double immunofluorescence was used to examine caspase-1 expression in microglia of hippocampus (Fig. 4d). Enhanced caspase-1 immunofluorescence was observed in Hypoxemia group, but not in Hypercapnia group compared with Sham group. HH group had the most intense caspase-1 fluorescence when compared with Hypoxemia group and Hypercapnia group. In rats given NAC treatment, caspase-1 fluorescence was noticeably attenuated (Fig. 4d).

Hypercapnia increased IL-1 β expression via inducing ROS overproduction in microglia in the hypoxic hippocampus

Significant interaction effects were observed between hypercapnia treatment and hypoxia treatment ($P < 0.01$) (Fig. 5b). Simple effects analyses found increased protein expression levels of IL-1 β in Hypoxemia group ($P < 0.01$), but not in Hypercapnia group ($P > 0.05$) compared with Sham group. HH group showed the highest expression levels of IL-1 β in comparison with Hypoxemia group ($P < 0.01$) and Hypercapnia group ($P < 0.01$) (Fig. 5c). Additionally, the protein expression of IL-1 β was significantly suppressed with NAC pretreatment (150 mg/kg) in rats ($P < 0.01$) (Fig. 5c). Double immunofluorescence was used to examine IL-1 β expression in microglia of hippocampus (Fig. 5d). Enhanced IL-1 β immunofluorescence was observed in Hypoxemia group, but not in Hypercapnia group compared with Sham group. HH group had the strongest IL-1 β fluorescence as compared with Hypoxemia group and Hypercapnia group. IL-1 β fluorescence was evidently reduced in rats given NAC pretreatment (Fig. 5d).

15% CO₂ increased the oxygen consumption rate (OCR) in hypoxic BV-2 microglial cells

The OCR levels of BV-2 microglial cells were examined after the treatment with 0.2% O₂ and 15% CO₂ for 0 h, 6 h, 12 h, and 24 h. The OCR levels were the highest in 12 h group as compared with other groups (12 h vs 0 h: $P < 0.01$; 12 h vs 6 h: $P > 0.05$; 12 h vs 24 h: $P < 0.01$). Besides, the OCR levels were increased time-dependently and achieved a stationary phase at 18 min (Fig. 6a). In view of this, microglia was treated for 12 hours, and the OCR was examined at 18 min after the treatment (Fig. 6b, c). Significant interaction effects were observed between 0.2% O₂ treatment and 15% CO₂ treatment ($P < 0.01$) (Fig. 6b). Simple effects analyses found increased levels of OCR in Hypoxia group ($P < 0.01$), but not in HC group ($P > 0.05$) compared with Control group. Hypoxia + HC group had the highest levels of OCR as compared with Hypoxia group ($P < 0.01$) and HC group ($P < 0.01$) (Fig. 6c).

15% CO₂ induced overproduction of ROS in hypoxic BV-2 microglial cells

Enhanced ROS immunofluorescence was observed in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group had the strongest ROS fluorescence as compared with Hypoxia group and HC group. ROS fluorescence was obviously reduced with NAC pretreatment (2 mM) in BV-2 microglial cells (Fig. 7).

15% CO₂ enhanced NLRP3 inflammasome activation via inducing ROS overproduction in hypoxic BV-2 microglial cells

Significant interaction effects were observed between 0.2% O₂ treatment and 15% CO₂ treatment ($P < 0.01$) (Fig. 8b). Simple effects analyses found increased protein expression levels of caspase-1 in Hypoxia group ($P < 0.01$), but not in HC group ($P > 0.05$) compared with Control group. Hypoxia + HC group had the highest expression levels of caspase-1 as compared with Hypoxia group ($P < 0.01$) and HC group ($P < 0.01$) (Fig. 8c). Additionally, the protein expression of caspase-1 was significantly suppressed with NAC pretreatment (2 mM) in BV-2 microglial cells ($P < 0.01$) (Fig. 8c). Double immunofluorescence was used to examine caspase-1 expression in BV-2 microglial cells (Fig. 8d). Enhanced caspase-1 immunofluorescence was observed in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group had the strongest caspase-1 fluorescence as compared with Hypoxia group and HC group. Of note, caspase-1 fluorescence was evidently reduced with NAC pretreatment (2 mM) in BV-2 microglial cells (Fig. 8d).

15% CO₂ increased IL-1 β expression via inducing ROS overproduction in hypoxic BV-2 microglial cells

Significant interaction effects were observed between 0.2% O₂ treatment and 15% CO₂ treatment ($P < 0.01$) (Fig. 9b). Simple effects analyses found increased protein expression levels of IL-1 β in Hypoxia group ($P < 0.01$), but not in HC group ($P > 0.05$) compared with Control group. Hypoxia + HC group had the highest expression levels of IL-1 β as compared with Hypoxia group ($P < 0.01$) and HC group ($P < 0.01$) (Fig. 9c). Additionally, the protein expression of IL-1 β was significantly suppressed with NAC pretreatment (2 mM) in BV-2 microglial cells ($P < 0.01$) (Fig. 9c). Double immunofluorescence confirmed IL-1 β expression in BV-2 microglial cells (Fig. 9d). Enhanced IL-1 β immunofluorescence was observed

in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group had the strongest IL-1 β fluorescence as compared with Hypoxia group and HC group. IL-1 β fluorescence was markedly suppressed with NAC pretreatment (2 mM) in BV-2 microglial cells (Fig. 9d).

Discussion

The present results have shown that hypercapnia enhanced the NLRP3 inflammasome activation via inducing ROS overproduction in hypoxia-activated microglia *in vitro* and *in vivo*. This was evident by the increased CERO₂, decreased PbtO₂, and increased expression levels of caspase-1 and IL-1 β in hypoxia-activated microglia cells.

The present rat and cell models of hypercapnia/hypoxemia were established as described in our previous study. In the models, PO₂ levels of the arterial blood and culture supernatant were maintained at 55–60 mmHg and pH levels of the arterial blood and culture supernatant at 7.20–7.25[9]. These are consistent with the change of hypoxemia and permissive hypercapnia in ARDS[25–28].

We reported in our previous study that hypercapnia-induced NLRP3 inflammasome activation in hypoxia-activated microglia could upregulate the expression of IL-1 β . It is well documented that IL-1 β promotes a cascade of inflammatory response in the central nervous system that can lead to the aggravation of neuronal injury[9]. Under hypoxic conditions, ROS is known to be critical for NLRP3 inflammasome activation[29–31]. Thus, the expression levels of caspase-1 and IL-1 β in the microglia in this study were determined, and the production of ROS in the hippocampus was also [evaluated](#). Significant interaction effects were observed on ROS production, caspase-1 and IL-1 β expression between hypercapnia treatment and hypoxia treatment. More importantly, we have shown that hypercapnia upregulated the expression of caspase-1 and IL-1 β in hypoxic hippocampus microglia via inducing ROS overproduction. Remarkably, caspase-1 and IL-1 β expression in hypoxic hippocampus microglia was downregulated when ROS was scavenged by NAC. These results indicate that hypercapnia enhanced the NLRP3 inflammasome activation via inducing ROS overproduction.

To determine the effect of hypercapnia on oxidative metabolism of the rat brain, cerebral oxygen extraction ratio (CERO₂) and partial pressure of brain tissue oxygen (PbtO₂) were examined. Hypoxemia, as a typical symptom of ARDS, decreases oxidative metabolism rate of the brain to avoid or reduce cerebral damage via preventing the PbtO₂ from dropping to a very low level[23]. In this study, we showed hypercapnia alone was not enough to increase or decrease the levels of PbtO₂ and CERO₂. In the first 1.5 hours, the levels of PbtO₂ were elevated by hypercapnia in the hypoxemic rats. This may be the reason why hypercapnia was neuroprotective in rats with transient global cerebral ischemia-reperfusion injury[32] and lateral fluid percussion injury[33]. However, hypercapnia significantly decreased the levels of PbtO₂ in the hypoxemic rats after 2 hours' ventilation. It was further evidenced by the increased levels of CERO₂ at 3 h after ventilation. These results suggest that hypercapnia is neuroprotective within a very

short period of time (1.5 h), but hypercapnia could aggravate neuronal functions injury over a protracted period via increasing $CERO_2$ and decreasing $PbtO_2$.

In vitro, to determine the effect of a high concentration of CO_2 on oxidative metabolism in hypoxic BV-2 microglial cells, the oxygen consumption rate (OCR) was evaluated. There was an interaction effect on the levels of OCR between 0.2% O_2 treatment and 15% CO_2 treatment. The high concentration of CO_2 treatment could increase the levels of OCR in hypoxic BV-2 microglial cells, which were consistent with that hypercapnia increased levels of $CERO_2$ in hypoxic rats. The results suggest that hypercapnia could intensify microglial hypoxia both *in vivo* and *in vitro*.

To ascertain if a high concentration of CO_2 would activate NLRP3 inflammasome via inducing ROS overproduction in hypoxia-activated BV-2 microglial cells, the production of ROS and expression of caspase-1 and IL-1 β was determined. The results were consistent with *in vivo* experiments. Significant interaction effects were observed on ROS production, caspase-1 and IL-1 β expression between 0.2% O_2 treatment and 15% CO_2 treatment. The high concentration of CO_2 upregulated the expression of caspase-1 and IL-1 β in hypoxia-activated microglia via inducing ROS overproduction, as evidenced by the downregulated expression of caspase-1 and IL-1 β when ROS was scavenged. All in all, the results support that a high concentration of CO_2 enhanced the NLRP3 inflammasome activation in hypoxia-activated BV-2 microglial cells via inducing and augmenting ROS overproduction.

In summary, the present results have demonstrated the underlying mechanism whereby hypercapnia can enhance the activation of NLRP3 inflammasome in hypoxic microglia. In this connection, hypercapnia was found to intensify the cerebral hypoxia via increasing $CERO_2$ and decreasing $PbtO_2$. As a consequence, ROS was overproduced in hypoxic microglial cells coupled with activation of NLRP3 inflammasome. Remarkably, caspase-1 and IL-1 β expression was downregulated when ROS was scavenged. Thus, hypercapnia-induced ROS overproduction and NLRP3 inflammasome activation in microglia may be a potential target to mitigate neuronal damage in the central nervous system such as that in the hippocampus.

Conclusions

Hypercapnia-induced ROS overproduction via intensifying cerebral hypoxia may activate the NLRP3 inflammasome and enhance IL-1 β release in hypoxia-activated microglia.

Abbreviations

ARDS: acute respiratory distress syndrome; CNS: central nervous system; IL-1 β : interleukin-1 beta; NLRP3: NLR family, pyrin domain-containing 3; $PbtO_2$: partial pressure of brain tissue oxygen; OCR: oxygen consumption rate; ROS: reactive oxygen species; Hb: hemoglobin concentration; PCO_2 : partial pressure of carbon dioxide; PaO_2 : partial pressure of artery blood oxygen; SaO_2 : saturation of artery blood

oxygen; PjVO₂: pressure of jugular vein blood oxygen; CaO₂: content of artery blood oxygen; CjVO₂: content of jugular vein blood oxygen; SjVO₂: saturation of jugular vein blood oxygen

Declarations

Ethics approval and consent to participate

The Experimental Animal Care and Use Committee at Jinan University approved all animal procedure protocols (No. 20171011001), which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All efforts were made to minimize the numbers of animals used and ensure minimal suffering.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DHG participated in making the animal model, assessed IL-1 β and caspase-1 expression in the hypoxic hippocampus microglia, collected data, and drafted the manuscript. LXS carried out the measurement of cerebral oxygen extraction ratio and partial pressure of brain tissue oxygen. LXQ conducted BV-2 microglial cells culture and treatment. WKR participated in evaluating ROS production of brain tissue and BV-2 microglial cells. LY participated in the evaluation of oxygen consumption rate evaluation *in vitro*. WMY performed statistical analysis. ZHK carried out the design of the study. All authors read and approved the final manuscript.

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Figures

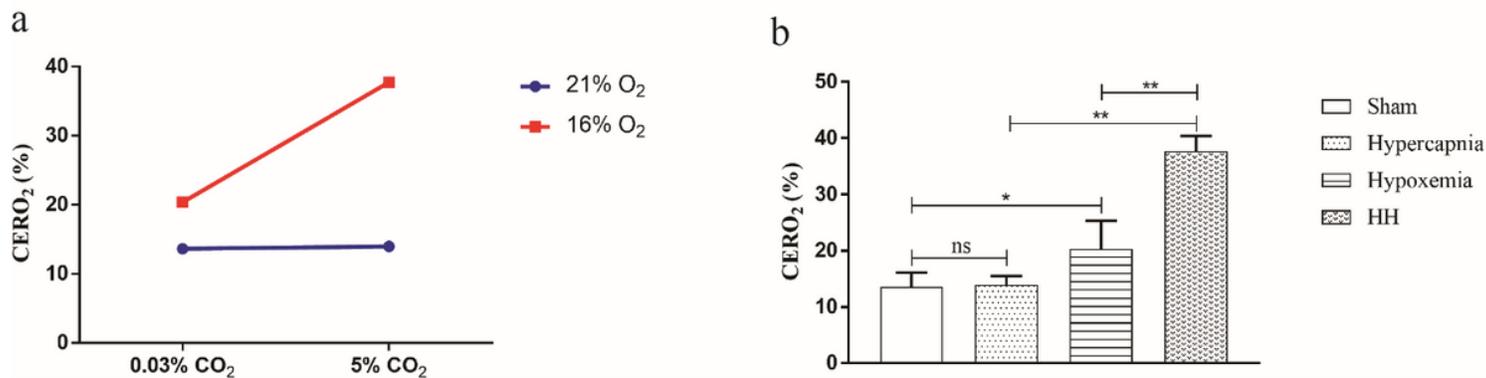


Figure 1

Hypercapnia increased CERO₂ in hypoxemic rats (n = 4). a There is an interaction effect between hypoxia treatment and hypercapnia treatment (P < 0.01). b Simple effects analyses show an increased CERO₂ in Hypoxemia group (* P < 0.05), but not in Hypercapnia group (ns P > 0.05) compared with Sham group. HH group has the highest CERO₂ levels as compared with Hypoxemia group (** P < 0.01) and Hypercapnia group (** P < 0.01). ns, non-significant; Sham group, sham-operated group; HH group, hypercapnia + hypoxemia group; CERO₂, cerebral oxygen extraction rate. The concentrations of O₂ and CO₂ in the air are 21% and 0.03%.

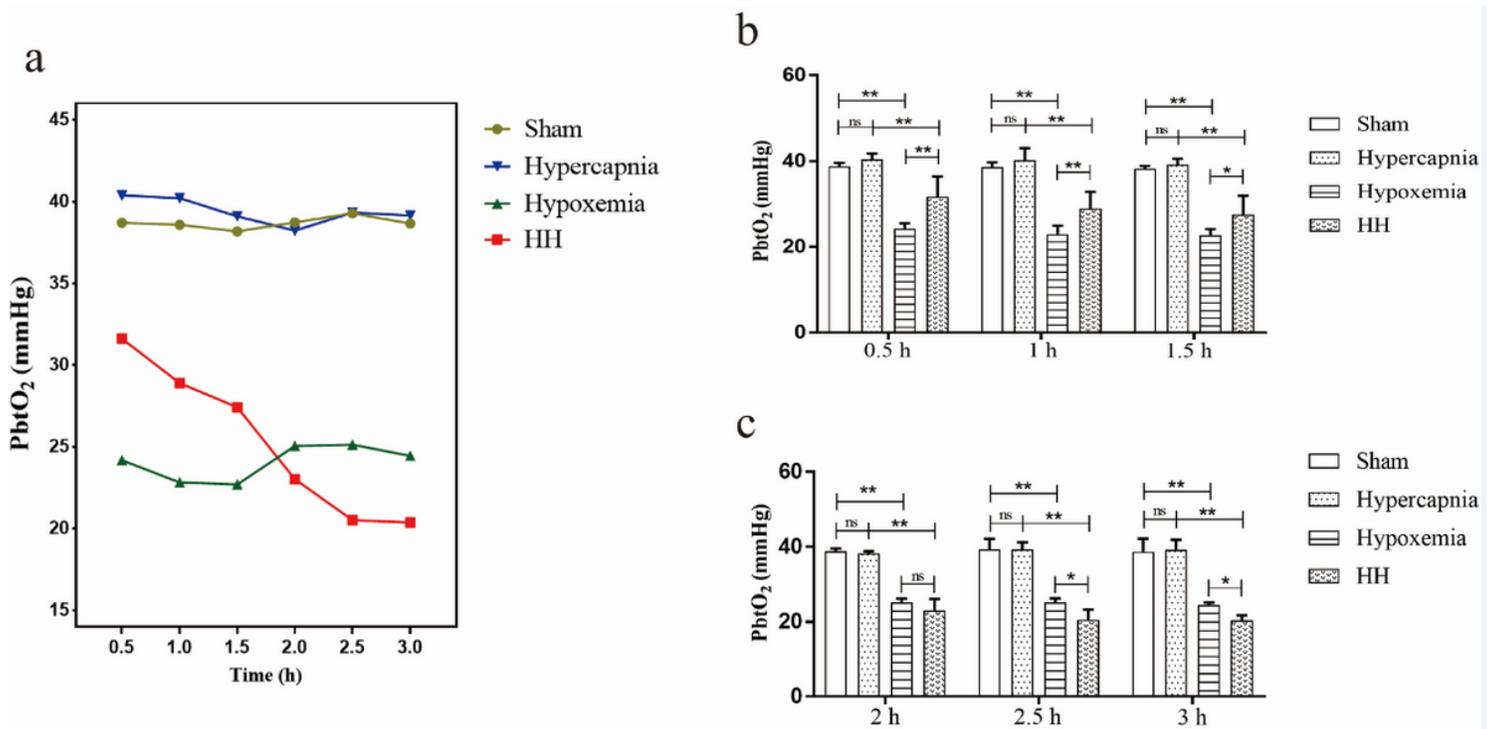


Figure 2

Hypercapnia decreased PbtO₂ in hypoxemic rats (n = 4). Graphs a illustrate the PbtO₂ at 0.5, 1, 1.5, 2, 2.5, and 3 h after ventilation in the rats. b, c Compared with the Sham group, PbtO₂ levels are decreased in Hypoxemia group (0.5 h - 3 h: P < 0.01), but not in Hypercapnia group (0.5 h - 3 h: P > 0.05). HH group has higher PbtO₂ levels as compared with Hypoxemia group in the first 1.5 hours (0.5 h: P < 0.01; 1 h: P < 0.01; 1.5 h: P < 0.05). On the contrary, HH group has lower PbtO₂ levels as compared with Hypoxemia group after 2 hours (2.5 h: P < 0.05; 3 h: P < 0.05). Sham group, sham-operated group; HH group, hypercapnia + hypoxemia group; PbtO₂, partial pressure of brain tissue oxygen.

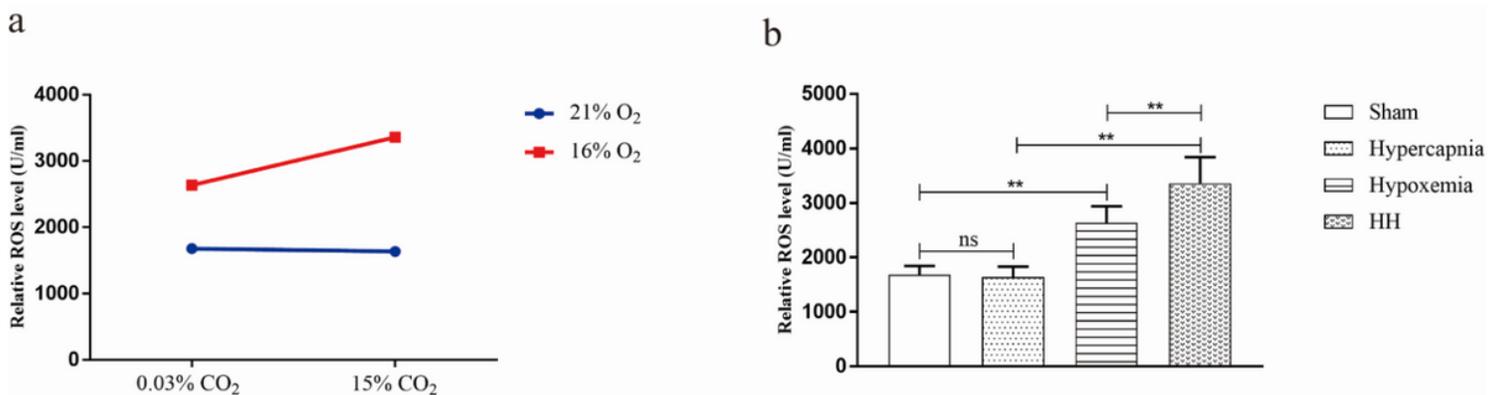


Figure 3

Hypercapnia induced overproduction of ROS in hypoxemic hippocampus (n = 4). a There is an interaction effect between hypoxia treatment and hypercapnia treatment (P < 0.05). b Simple effects analyses show increased levels of ROS in Hypoxemia group (** P < 0.01), but not in Hypercapnia group (ns P > 0.05) compared with Sham group. HH group has the highest ROS levels as compared with Hypoxemia group

(** P < 0.01) and Hypercapnia group (** P < 0.01). ns, non-significant; Sham group, sham-operated group; HH group, hypercapnia + hypoxemia group; ROS, reactive oxygen species. The concentrations of O₂ and CO₂ in the air are 21% and 0.03%.

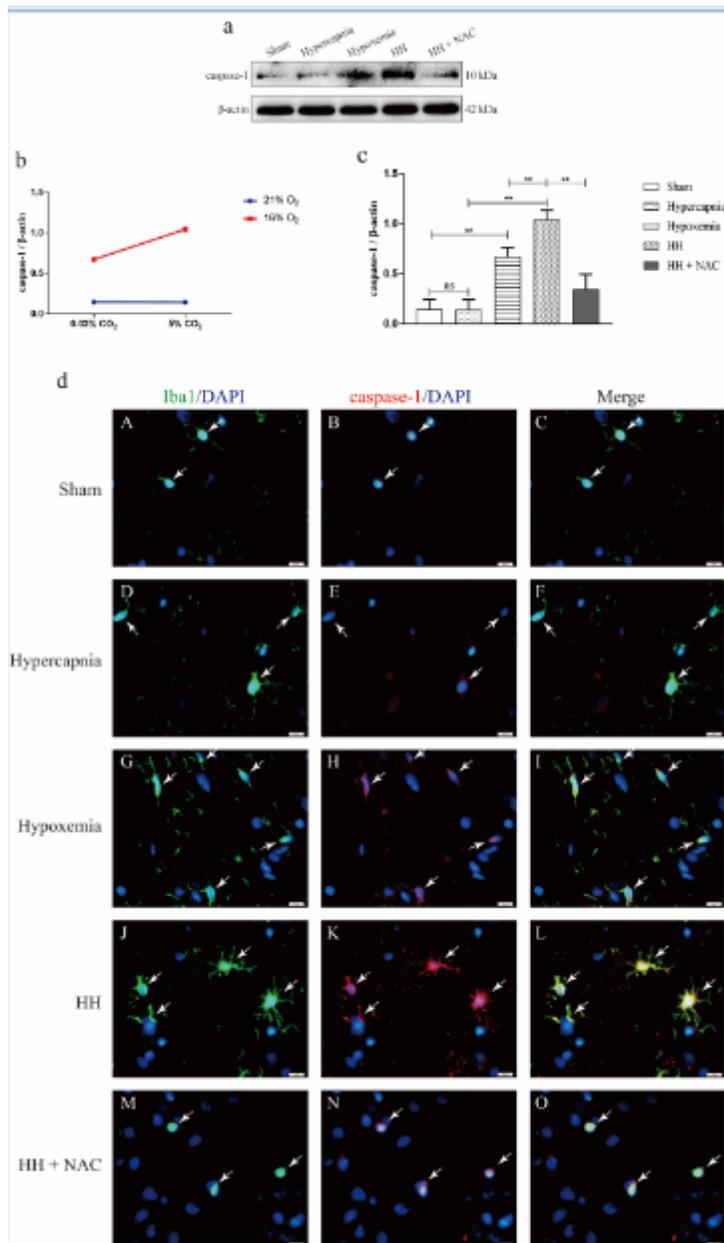


Figure 4

Hypercapnia enhanced NLRP3 inflammasome activation via inducing ROS overproduction in hypoxic microglia (n = 4). a Immunoreactive bands of caspase-1 (10 kDa) and β -actin (42 kDa). b There is an interaction effect between hypoxia treatment and hypercapnia treatment (P < 0.01). c Simple effects analyses show increased protein expression levels of caspase-1 in Hypoxemia group (** P < 0.01), but not in Hypercapnia group (ns P > 0.05) compared with Sham group. HH group has the highest expression levels of caspase-1 as compared with Hypoxemia group (** P < 0.01) and Hypercapnia group (** P < 0.01). The protein expression of caspase-1 is significantly suppressed with NAC pretreatment (150 mg/kg) in rats (** P < 0.01). d Immunofluorescence images showing the expression of Iba1+ microglia (A,

D, G, J, M, green), caspase-1 (B, E, H, K, N, red), and the co-localization of caspase-1 and microglia (C, F, I, L, O). Enhanced caspase-1 immunofluorescence is evident in Hypoxemia group, but not in Hypercapnia group compared with Sham group. HH group emits the strongest caspase-1 fluorescence as compared with Hypoxemia group and Hypercapnia group. Caspase-1 fluorescence is markedly attenuated in rats given NAC pretreatment. Scale bars: 10 μ m. ns, non-significant; Sham group, sham-operated group; HH group, hypercapnia + hypoxemia group. The concentrations of O₂ and CO₂ in the air are 21% and 0.03%.

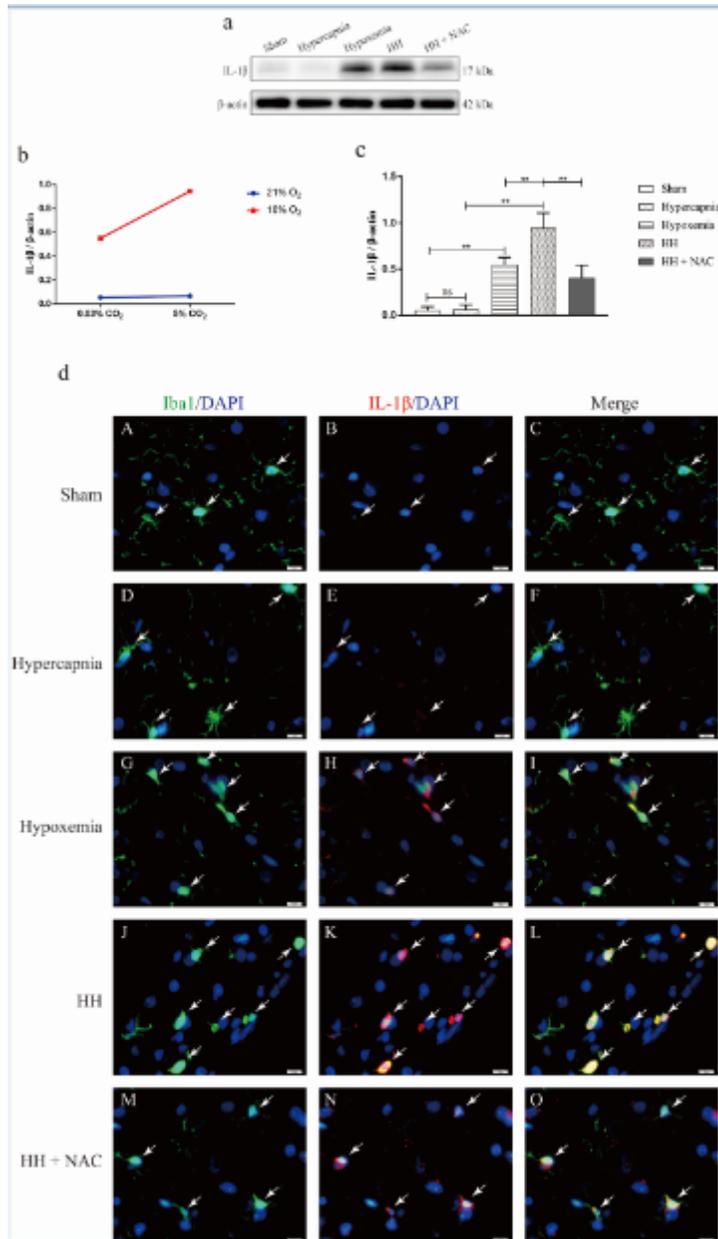


Figure 5

Hypercapnia increased IL-1 β expression via inducing ROS overproduction in hypoxic microglia (n = 4). a Immunoreactive bands of IL-1 β (17 kDa) and β -actin (42 kDa). b There is an interaction effect between hypoxia treatment and hypercapnia treatment (P < 0.01). c Simple effects analyses show increased protein expression levels of IL-1 β in Hypoxemia group (**P < 0.01), but not in Hypercapnia group (ns P > 0.05) compared with Sham group. HH group has the highest expression levels of IL-1 β as compared with

Hypoxemia group (** P < 0.01) and Hypercapnia group (** P < 0.01). The protein expression of IL-1 β is significantly suppressed with NAC pretreatment (150 mg/kg) in rats (** P < 0.01). d Immunofluorescence images showing the expression of Iba1+ microglia (A, D, G, J, M, green), IL-1 β (B, E, H, K, N, red), and the co-localization of IL-1 β and microglia (C, F, I, L, O). Enhanced IL-1 β immunofluorescence is evident in Hypoxemia group, but not in Hypercapnia group compared with Sham group. HH group has the strongest IL-1 β fluorescence as compared with Hypoxemia group and Hypercapnia group. IL-1 β fluorescence is markedly reduced in rats given NAC pretreatment. Scale bars: 10 μ m. IL-1 β , interleukin-1 beta; ns, non-significant; Sham group, sham-operated group; HH group, hypercapnia + hypoxemia group. The concentrations of O₂ and CO₂ in the air are 21% and 0.03%.

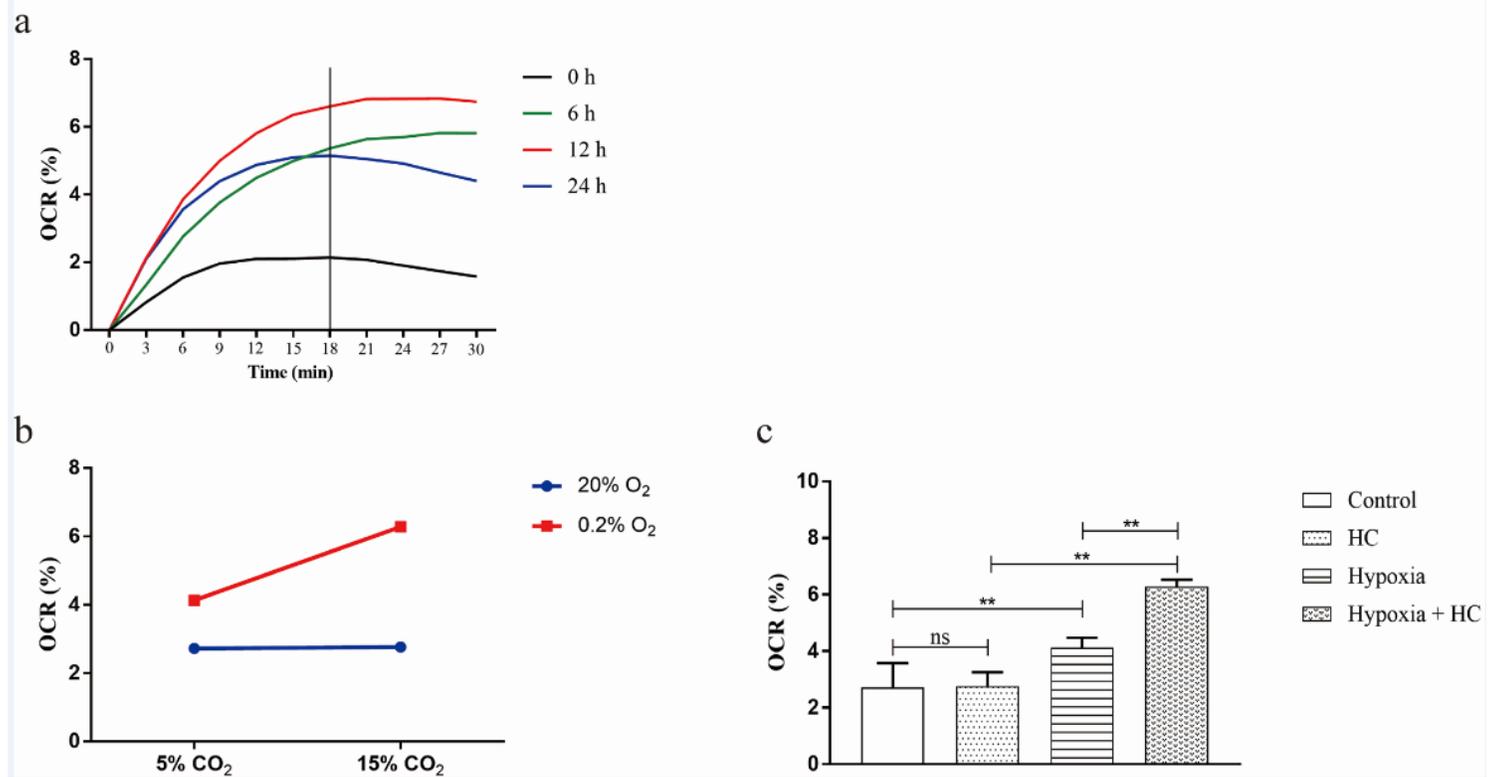


Figure 6

15% CO₂ increased the OCR in hypoxic BV-2 cells (n = 4). a The OCR levels are the highest in 12 h group as compared with other groups (12 h vs 0 h: P < 0.01; 12 h vs 6 h: P > 0.05; 12 h vs 24 h: P < 0.01). The OCR levels are increased time-dependently and achieved a stationary phase at 18 min. So the microglia cells were treated for 12 hours, and the OCR was examined at 18 min after the treatment, as shown in graph b and c. b There is an interaction effect between 0.2% O₂ treatment and 15% CO₂ treatment (P < 0.01). c Simple effects analyses show increased levels of OCR in Hypoxia group (** P < 0.01), but not in HC group (ns P > 0.05) compared with Control group. Hypoxia + HC group has the highest levels of OCR as compared with Hypoxia group (** P < 0.01) and HC group (** P < 0.01). ns, non-significant; HC group, high concentration of carbon dioxide group.

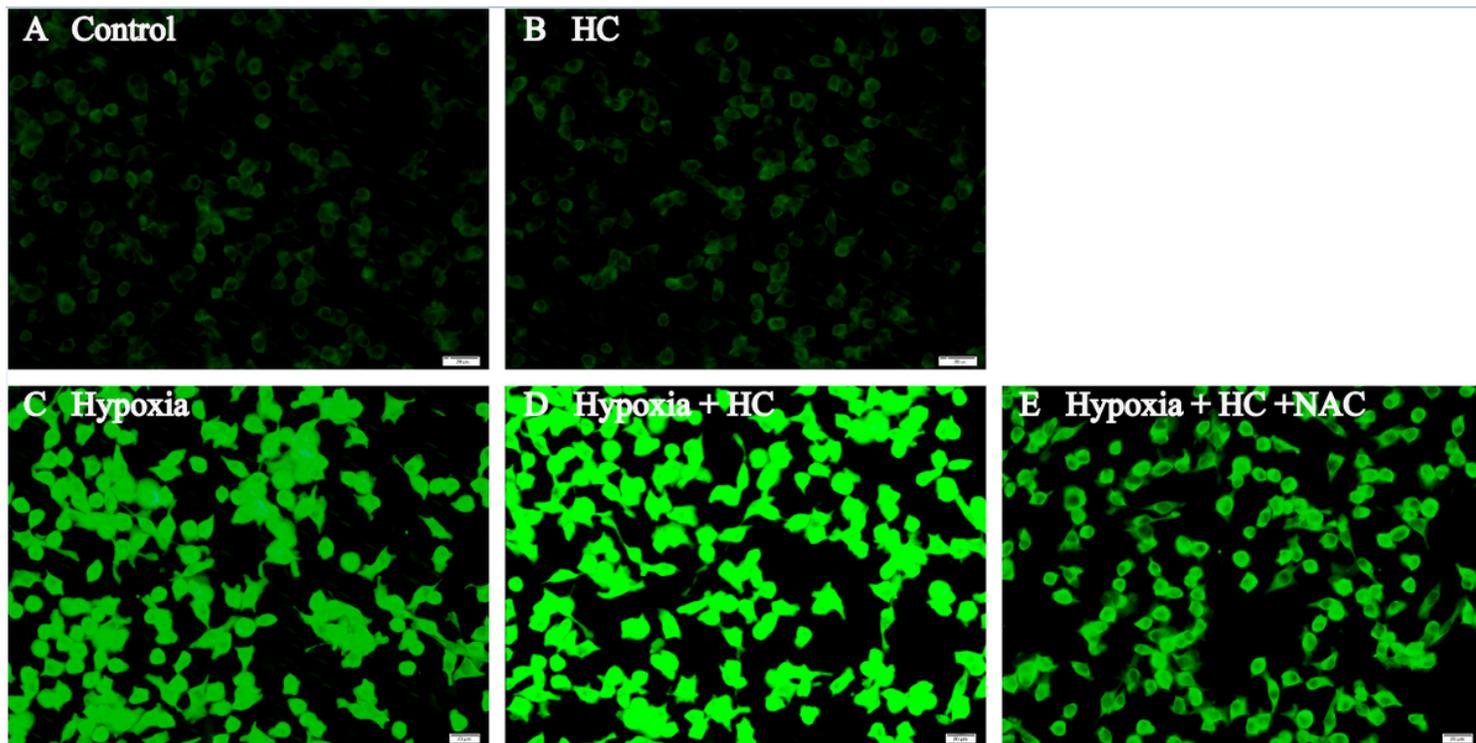


Figure 7

15% CO₂ induced overproduction of ROS in hypoxic BV-2 cells (n = 4). Immunofluorescence images showing the production of ROS (A, B, C, D, E, green). Enhanced ROS immunofluorescence is evident in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group exhibits the most intense ROS fluorescence as compared with Hypoxia group and HC group. ROS fluorescence is noticeably reduced with NAC pretreatment (2 mM) in BV-2 microglial cells. Scale bars: 20 μ m. ROS, reactive oxygen species; HC group, high concentration of carbon dioxide group.

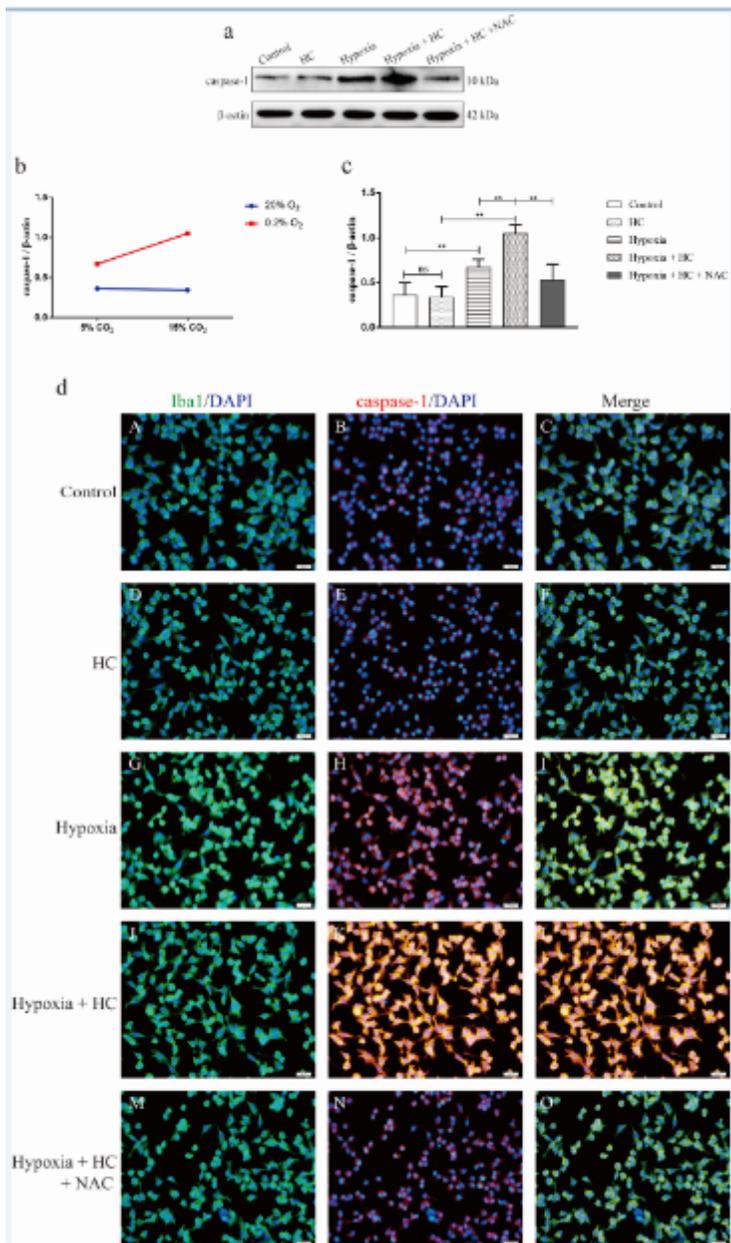


Figure 8

15% CO_2 enhanced NLRP3 inflammasome activation via inducing ROS overproduction in hypoxic BV-2 cells. **a** Immunoreactive bands of caspase-1 (10 kDa) and β -actin (42 kDa). **b** There is an interaction effect between 0.2% O_2 treatment and 15% CO_2 treatment ($P < 0.01$, $n = 4$). **c** Simple effects analyses show increased protein expression levels of caspase-1 in Hypoxia group ($** P < 0.01$), but not in HC group ($ns P > 0.05$) compared with Control group. Hypoxia + HC group shows the highest expression levels of caspase-1 in comparison with Hypoxia group ($** P < 0.01$) and HC group ($** P < 0.01$). Additionally, the protein expression of caspase-1 is significantly suppressed with NAC pretreatment (2 mM) in BV-2 microglial cells ($** P < 0.01$). **d** Immunofluorescence images showing the expression of Iba1+ microglia (A, D, G, J, M, green), caspase-1 (B, E, H, K, N, red), and the co-localization of caspase-1 and microglia (C, F, I, L, O). Enhanced caspase-1 immunofluorescence is evident in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group shows the strongest caspase-1 fluorescence as

compared with Hypoxia group and HC group. Caspase-1 fluorescence is drastically reduced with NAC pretreatment (2 mM) in BV-2 microglial cells. Scale bars: 10 μ m. ns, non-significant; HC group, high concentration of carbon dioxide group.

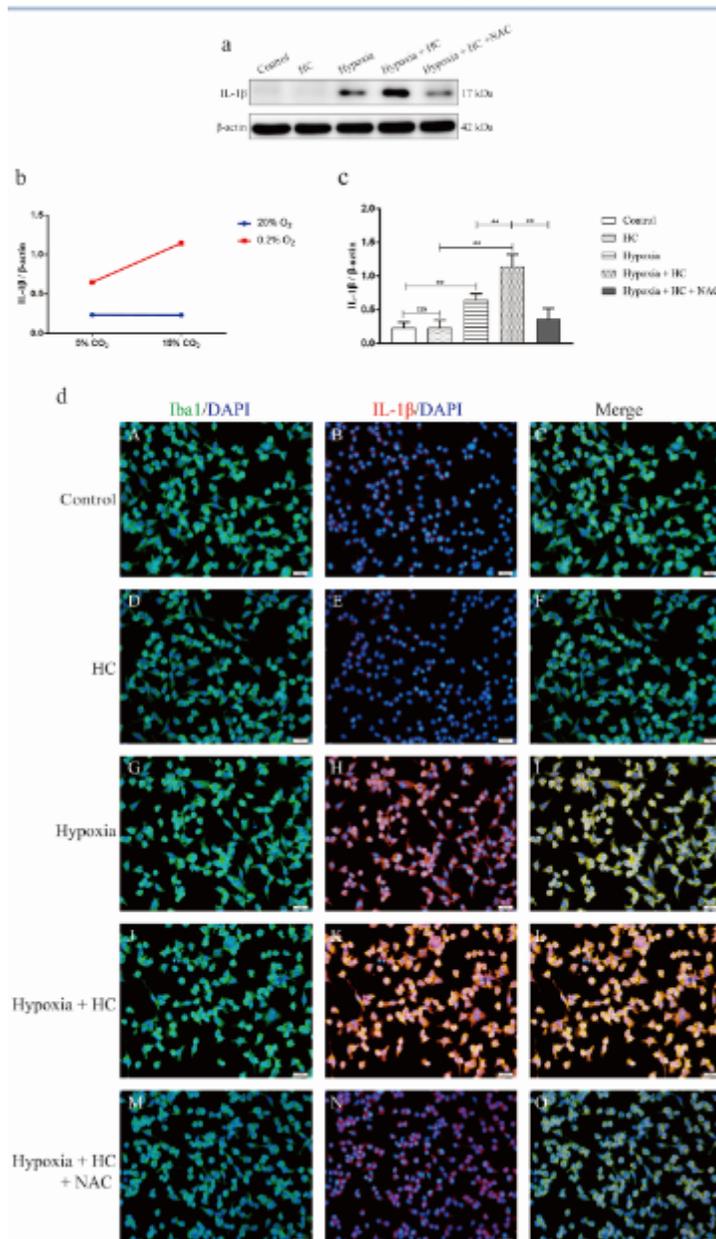


Figure 9

15% CO₂ increased IL-1 β expression via inducing ROS overproduction in hypoxic BV-2 cells. a Immunoreactive bands of IL-1 β (17 kDa) and β -actin (42 kDa). b There is an interaction effect between 0.2% O₂ treatment and 15% CO₂ treatment ($P < 0.01$, $n = 4$). c Simple effects analyses show increased protein expression levels of IL-1 β in Hypoxia group (** $P < 0.01$), but not in HC group (ns $P > 0.05$) compared with Control group. Hypoxia + HC group shows the highest expression levels of IL-1 β as compared with Hypoxia group (** $P < 0.01$) and HC group (** $P < 0.01$) (Fig. 9c). Additionally, the protein expression of IL-1 β is significantly suppressed with NAC pretreatment (2 mM) in BV-2 microglial cells (** $P < 0.01$). d Immunofluorescence images showing Iba1+ microglia (A, D, G, J, M, green), IL-1 β labeling (B,

E, H, K, N, red), and their co-localization (C, F, I, L, O). Enhanced IL-1 β immunofluorescence is evident in Hypoxia group, but not in HC group compared with Control group. Hypoxia + HC group displays the strongest IL-1 β fluorescence when compared with Hypoxia group and HC group. IL-1 β fluorescence is markedly reduced with NAC pretreatment (2 mM) in BV-2 microglial cells. Scale bars: 10 μ m. IL-1 β , interleukin-1 beta; ns, non-significant; HC group, high concentration of carbon dioxide group.

Supplementary Files

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