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# Treatment with 7% and 10% $CO_2$ enhanced expression of IL-1β, TNF-α, and IL-6 in hypoxic cultures of human whole blood

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## Abstract

**Objective:** This study investigated whether hypercapnia influenced the inflammatory response of hypoxic blood.

**Methods:** Human whole blood was cultured with 0.2% oxygen ($O_2$) and treated with 5%, 7%, or 10% carbon dioxide ($CO_2$). Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 were evaluated in whole blood cultures. Reactive oxygen species (ROS) production and expression levels of caspase-1 and IL-1β were evaluated in THP-1 monocytic cells.

**Results:** IL-1β, TNF-α, and IL-6 levels were higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group. The hypoxia + 10% $CO_2$ group had the highest IL-1β, TNF-α, and IL-6 levels, compared with the hypoxia + 7% $CO_2$ and hypoxia + 5% $CO_2$ groups. Expression levels of IL-1β, TNF-α, and IL-6 were significantly negatively correlated with pH levels in the cell culture medium. Treatment with 7% and 10% $CO_2$ increased the production of ROS and the expression of caspase-1 and IL-1β in hypoxia-activated THP-1 cells.

**Conclusions:** High levels of $CO_2$ treatment increased expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures. High levels of $CO_2$-induced ROS overproduction and NLRP3 inflammasome activation in monocytes may comprise a target to mitigate the inflammatory response of hypoxic blood.

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**Keywords**
Hypercapnia, hypoxemia, acute respiratory distress syndrome, inflammation, caspase 1, reactive oxygen species, monocytes, interleukin-6, tumor necrosis factor-alpha, interleukin-1

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## Introduction

Refractory hypoxemia is the main clinical manifestation of acute respiratory distress syndrome (ARDS).[1,2] Lower tidal ventilation is available for routine clinical application, which enables alleviation of hypoxemia, as well as avoidance of ventilator-induced lung injury.[3,4] This ventilation strategy inevitably leads to hypercapnia—elevated levels of carbon dioxide ($CO_2$) in the blood.[5,6] A lower tidal ventilation strategy exerts some beneficial effects on respiratory systems, which can reduce pulmonary morbidity for patients with ARDS.[5,7,8] However, hypercapnia is a non-physiological state for the human body; thus far, it remains controversial whether hypercapnia is detrimental or protective for patients with ARDS.[9,10]

As the main clinical symptom of ARDS, hypoxemia can trigger secretion of inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) in the blood.[11] In addition, the blood-mediated inflammatory response can cause injury to critical organs.[12] There are close links between the degree of hypoxemia and mortality in patients with ARDS, as well as between the duration of hypoxemia and mortality in those patients.[13] For patients with both hypoxemia and hypercapnia, the molecular mechanism of the inflammatory response may be complex. Hypercapnia has been reported to attenuate endotoxin-induced inflammation by inhibiting activation of nuclear factor-κB.[14,15] However, under hypoxic conditions, there may be differences in the underlying mechanism whereby hypercapnia exerts its effects on the blood-mediated inflammatory response.

Our previous study revealed that hypercapnia-induced activation of the NLR family, pyrin domain-containing 3 protein (NLRP3) inflammasome could upregulate the expression of IL-1β in hypoxia-activated microglia.[16] Moreover, IL-1β has been shown to promote the central nervous system cascade inflammatory response.[17,18] It is unknown whether hypercapnia exerts any effects on the peripheral inflammation of hypoxic blood. Here, we hypothesized that hypercapnia might enhance the inflammation of hypoxic blood, specifically by increasing IL-1β, TNF-α, and IL-6 expression in hypoxic blood.

## Materials and methods

### *Ethics and consent*

The study was approved by the Research Ethics Committee of Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China (Approval no. GDREC2018424H(R1)). All participants provided written informed consent for inclusion in the study.

### *Human whole blood cultures and treatment*

Whole blood was collected from six healthy male volunteers (mean age, $38 \pm 9.9$ years).

None of the volunteers had a history of hematologic disorders, transplantations, cancers, autoimmune diseases, infection, or immunosuppressive drug use. Whole blood was cultured as reported previously.[14] Briefly, 2 mL of whole blood were collected from each volunteer. Whole blood samples were cultured with 18 mL Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA; cat. no. R2405) supplemented with 10% human serum (Gemini Bio-Products, Calabasas, CA, USA; cat. no. H91S001) and 1% penicillin-streptomycin solution (Roche, Basel, Switzerland; cat. no. 11074440001) at 37°C in a humidified incubator with 5% $CO_2$/95% air.

Blood samples were randomly divided into four groups: control (exposed to 20% $O_2 + 5\%$ $CO_2$ for 24 hours), hypoxia + 5% $CO_2$ (exposed to 5% $CO_2 + 0.2\%$ $O_2$ for 24 hours), hypoxia + 7% $CO_2$ (exposed to 7% $CO_2 + 0.2\%$ $O_2$ for 24 hours), and hypoxia + 10% $CO_2$ (exposed to 10% $CO_2 + 0.2\%$ $O_2$ for 24 hours). The partial pressure of oxygen ($PO_2$), partial pressure of carbon dioxide ($PCO_2$), and pH of the medium were measured at 24 hours ($n = 6$ per group) using a Blood Gas/Electrolyte Analyzer (Model 5700, Instrumentation Laboratory, San Diego, CA, USA).

## *Evaluation of IL-1β, TNF-α, and IL-6 expression levels in whole blood cultures*

IL-1β, TNF-α, and IL-6 levels were evaluated using enzyme-linked immunosorbent assay kits (all from Abcam, Cambridge, MA, USA; IL-1β: cat. no. ab46052; TNF-α: cat. no. ab181421; IL-6: cat. no. ab178013), in accordance with the manufacturers' instructions. Briefly, samples and standards were added to plate wells that had been coated with horseradish peroxidase-labeled anti-IL-1β, anti-TNF-α, and anti-IL-6 antibodies. A 3,3',5,5'-tetramethylbenzidine substrate solution was then added to the wells. Subsequently, stop buffer was added, and the optical density at 450 nm was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations of IL-1β, TNF-α, and IL-6 in the samples were determined by comparing the optical density of the samples with the optical density of the standard curve. This experiment was repeated six times.

## *Human THP-1 cells: culture and treatment*

Monocytes are reportedly the main cellular source of proinflammatory cytokines.[19,20] Thus, the THP-1 human monocytic cell line was used in the present study; these cells were purchased form American Type Culture Collection (Manassas, VA, USA). THP-1 cells were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen; cat. no. 16140071) and 1% penicillin-streptomycin solution at 37°C in a 5% $CO_2$ incubator. THP-1 monocytes were cultured in six-well plates ($1 \times 10^6$ cells/mL, 2 mL) for 48 hours in the presence of 100 nM phorbol 12-myristate 13-acetate,[21] which stimulated differentiation into macrophages. Macrophages were randomly divided into six groups: control (exposed to 5% $CO_2 + 20\%$ $O_2$), hypoxia + 5% $CO_2$ (exposed to 5% $CO_2 + 0.2\%$ $O_2$), hypoxia + 7% $CO_2$ (exposed to 7% $CO_2 + 0.2\%$ $O_2$), hypoxia + 10% $CO_2$ (exposed to 10% $CO_2 + 0.2\%$ $O_2$), hypoxia + 10% $CO_2$ + N-acetyl-L-cysteine (NAC), and hypoxia + 10% $CO_2$ + Z-YVAD-FMK. Cells in the hypoxia + 10% $CO_2$ + NAC and hypoxia + 10% $CO_2$ + Z-YVAD-FMK groups were respectively treated with an ROS scavenger, 25 mM NAC[22–24] (MedChemExpress, Monmouth, NJ, USA; cat. no. HY-B0215), and a pan-caspase inhibitor, 10 μM Z-YVAD-FMK[25,26] (ApexBio, Boston, MA, USA;

cat. no. A8955), for 30 minutes before exposure to 10% $CO_2 + 0.2\%$ $O_2$.

### *Measurement of reactive oxygen species (ROS)*

ROS production in macrophages was evaluated using a ROS assay kit (BestBio, Shanghai, China; cat. no. BB-4705-2), in accordance with the manufacturers' instructions. Briefly, DCFH-DA was diluted (1:1500) with RPMI-1640 medium without fetal bovine serum. Coverslips with adherent cells were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum. The medium was changed to diluted DCFH-DA (2 mL/well) after treatment with different concentrations of $CO_2$ and $O_2$; cells were incubated in this medium for 20 minutes at 37°C with 5% $CO_2$. Coverslips were then washed using RPMI-1640 medium without fetal bovine serum. Finally, coverslips were mounted with a fluorescent mounting medium and detected using a fluorescence microscope (Olympus DP73 Microscope, Olympus, Tokyo, Japan). This experiment was repeated four times.

### *Western blotting*

In accordance with the manufacturer's instructions, total protein extracts from cells were prepared using a Total Protein Extraction Kit (BestBio Science, Shanghai, China; cat. no. BB-3101-100T). Total protein concentrations were determined using a Bioepitope Bicinchoninic Acid Protein Assay Kit (Bioworld Technology Co., Ltd, Nanjing, China; cat. no. 23227). Protein samples were separated on polyacrylamide-SDS gels and transferred onto nitrocellulose membranes. After membranes had been blocked with 5% non-fat dry milk in Tris-buffered saline for 1 hour, they were incubated overnight at 4°C with the following primary antibodies: anti-caspase-1 (rabbit monoclonal IgG, 1:1000, Abcam; cat. no. ab207802) and anti-IL-1β (rabbit polyclonal IgG, 0.2 μg/mL, Abcam; cat. no. ab9722). After membranes had been washed three times in Tris-buffered saline with Tween-20, they were incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA; cat. no. #7074) for 45 minutes at room temperature. Immunoblots were developed using an enhanced chemiluminescence detection system (ImageQuant LAS 500, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Band intensities were quantified using ImageJ 1.39u software (National Institutes of Health, Bethesda, MD, USA). This experiment was repeated four times.

### *Statistical analysis*

Statistical analysis was performed using SPSS Statistics, version 19.0 (IBM Corp., Armonk, NY, USA). All values are expressed as means ± standard deviations. One-way analysis of variance was used to analyze univariate-factor measurement data. Correlations between pH and each of the following factors were analyzed with Spearman's rank correlation coefficient: IL-1β, TNF-α, and IL-6. Differences were considered statistically significant when $P < 0.05$.

## Results

### *$PO_2$, $PCO_2$, and pH levels in whole blood culture medium*

Compared with the control group, 0.2% $O_2$ treatment reduced $PO_2$ levels in the medium of the hypoxia + 5% $CO_2$ ($P < 0.01$), hypoxia + 7% $CO_2$ ($P < 0.01$), and hypoxia + 10% $CO_2$ groups ($P < 0.01$). $PO_2$ levels did not significantly differ among

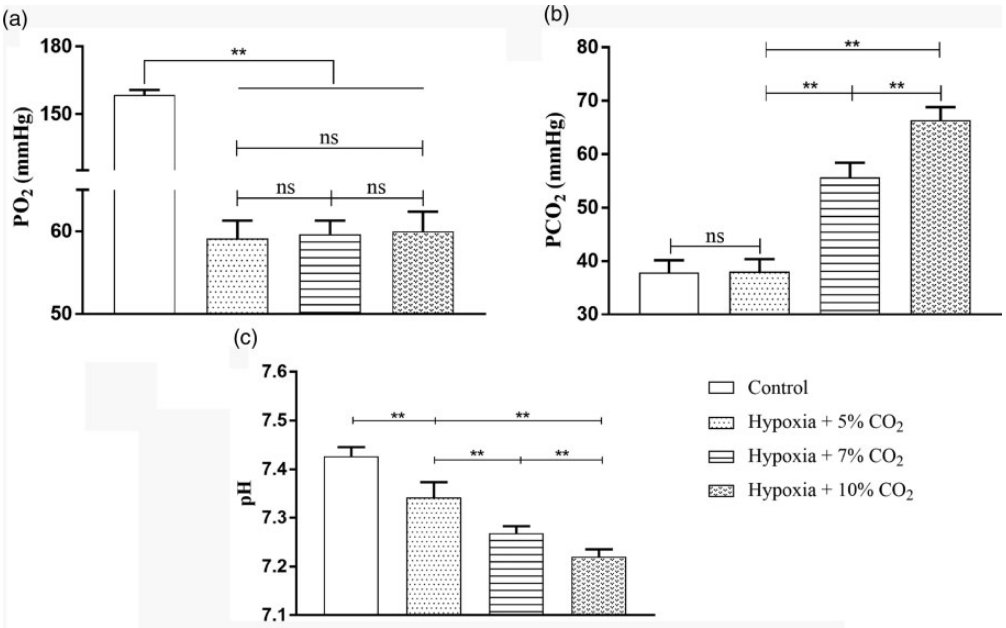


**Figure 1.** $PO_2$ (a), $PCO_2$ (b), and pH (c) levels in whole blood culture medium at 24 hours (n = 6 per group). $^{**}P < 0.01$. Exposures were as follows: control (20% $O_2$ + 5% $CO_2$), hypoxia + 5% $CO_2$ (5% $CO_2$ + 0.2% $O_2$), hypoxia + 7% $CO_2$ (7% $CO_2$ + 0.2% $O_2$), and hypoxia + 10% $CO_2$ (10% $CO_2$ + 0.2% $O_2$). Abbreviations: $PCO_2$, partial pressure of carbon dioxide; $PO_2$, partial pressure of artery blood oxygen

0.2% $O_2$-treated groups (Figure 1a; Table 1); $PCO_2$ levels also did not significantly differ between the hypoxia + 5% $CO_2$ and control groups. $PCO_2$ levels were much higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group ($P < 0.01$). Notably, the hypoxia + 10% $CO_2$ group had the highest $PCO_2$ levels, compared with the hypoxia + 7% $CO_2$ ($P < 0.01$) and hypoxia + 5% $CO_2$ groups ($P < 0.01$) (Figure 1b; Table 1). pH levels were much lower in the hypoxia + 5% $CO_2$ group than in the control group ($P < 0.01$); pH levels were also much lower in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group ($P < 0.01$). Notably, the hypoxia + 10% group had the lowest pH levels, compared with the hypoxia + 7% $CO_2$ ($P < 0.01$) and hypoxia + 5% $CO_2$ groups ($P < 0.01$) (Figure 1c; Table 1).

### *Higher levels of $CO_2$ treatment increased IL-1β, TNF-α, and IL-6 expression levels in hypoxic whole blood cultures*

IL-1β levels were much higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group ($P < 0.05$); the hypoxia + 10% $CO_2$ group had the highest IL-1β levels, compared with the hypoxia + 7% $CO_2$ ($P < 0.05$) and hypoxia + 5% $CO_2$ groups ($P < 0.01$) (Figure 2a; Table 2). TNF-α levels were much higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group ($P < 0.05$); the hypoxia + 10% $CO_2$ group had the highest TNF-α levels, compared with the hypoxia + 7% $CO_2$ ($P < 0.05$) and hypoxia + 5% $CO_2$ groups ($P < 0.01$) (Figure 2b; Table 2). IL-6 levels were much higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group ($P < 0.05$); the

**Table 1.** $PO_2$, $PCO_2$, and pH levels in whole blood culture medium (n = 6 per group)

| | $PO_2$ (mmHg) | $PCO_2$ (mmHg) | pH |
|---|---|---|---|
| Control | $158.50 \pm 2.17$ | $37.83 \pm 2.32$ | $7.43 \pm 0.02$ |
| Hypoxia + 5% $CO_2$ | $59.17 \pm 2.14^{*}$ | $38.00 \pm 2.37$ | $7.34 \pm 0.03$ |
| Hypoxia + 7% $CO_2$ | $59.67 \pm 1.63^{*}$ | $55.67 \pm 2.73^{* \#}$ | $7.27 \pm 0.01^{* \#}$ |
| Hypoxia + 10% $CO_2$ | $60.00 \pm 2.37^{*}$ | $66.33 \pm 2.50^{* \#}$ | $7.22 \pm 0.02^{* \#}$ |

Compared with the control group: $^{*}P < 0.01$; compared with the hypoxia + 5% $CO_2$ group: $^{\#}P < 0.01$.
Abbreviations: $PCO_2$, partial pressure of carbon dioxide; $PO_2$, partial pressure of artery blood oxygen; pH, potential of hydrogen.

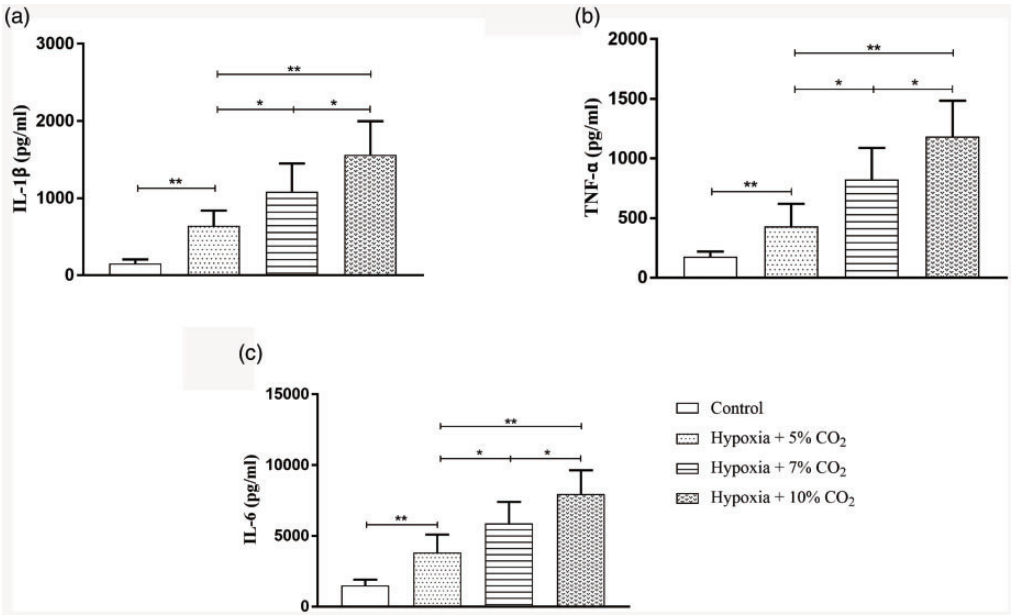


**Figure 2.** Higher levels of $CO_2$ treatment increased (a) IL-1$\beta$, (b) TNF-$\alpha$, and (c) IL-6 expression in hypoxic whole blood cultures (n = 6 per group; evaluated by enzyme-linked immunosorbent assay). $^{*}P < 0.05$, $^{**}P < 0.01$. Exposures were as follows: control (20% $O_2$ + 5% $CO_2$), hypoxia + 5% $CO_2$ (5% $CO_2$ + 0.2% $O_2$), hypoxia + 7% $CO_2$ (7% $CO_2$ + 0.2% $O_2$), and hypoxia + 10% $CO_2$ (10% $CO_2$ + 0.2% $O_2$).
Abbreviations: IL-1$\beta$, interleukin-1 beta; TNF-$\alpha$, tumor necrosis factor alpha; IL-6, interleukin-6

**Table 2.** Effects of $CO_2$ treatment on expression levels of IL-1$\beta$, TNF-$\alpha$, and IL-6 in hypoxic whole blood cultures (n = 6 per group)

| | IL-1$\beta$ (pg/mL) | TNF-$\alpha$ (pg/mL) | IL-6 (pg/mL) |
|---|---|---|---|
| Hypoxia + 5% $CO_2$ | $642.33 \pm 196.58$ | $431.50 \pm 188.32$ | $3829.33 \pm 1257.81$ |
| Hypoxia + 7% $CO_2$ | $1082.67 \pm 368.24^{\$}$ | $822.17 \pm 266.74^{\$}$ | $5869.67 \pm 1529.56^{\$}$ |
| Hypoxia + 10% $CO_2$ | $1562.83 \pm 435.32^{\# \&}$ | $1185.67 \pm 299.16^{\# \&}$ | $7952.33 \pm 1692.40^{\# \&}$ |

Compared with the hypoxia + 5% $CO_2$ group: $^{\$}P < 0.05$, $^{\#}P < 0.01$; compared with the hypoxia + 7% $CO_2$ group: $^{\&}P < 0.05$.
Abbreviations: IL-1$\beta$, interleukin-1 beta; TNF-$\alpha$, tumor necrosis factor alpha; IL-6, interleukin-6.

hypoxia + 10% $CO_2$ group had the highest IL-6 levels, compared with the hypoxia + 7% $CO_2$ ($P < 0.05$) and hypoxia + 5% $CO_2$ groups ($P < 0.01$) (Figure 2c; Table 2).

### *IL-1β, TNF-α, and IL-6 expression levels were negatively correlated with pH level*

Expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures were significantly negatively correlated with pH levels in the medium (IL-1β: $\rho = -0.735$, $P < 0.01$; TNF-α: $\rho = -0.810$, $P < 0.01$; IL-6: $\rho = -0.805$, $P < 0.01$) (Figure 3).

### *Higher levels of $CO_2$ treatment induced overproduction of ROS in hypoxic THP-1 cells*

The fluorescence intensity of ROS in THP-1 cells was noticeably increased by exposure to hypoxia, compared with the control group (Figure 4a, b). Fluorescence in hypoxic cells was further enhanced by treatment with 7% or 10% $CO_2$ (Figure 4c, d). In contrast, ROS fluorescence was markedly reduced in cells that had been pretreated with NAC (Figure 4e).

### *High levels of $CO_2$ exposure led to enhanced NLRP3 inflammasome activation and elevated IL-1β expression in hypoxic THP-1 cells*

To explore the effects of high levels of $CO_2$ on NLRP3 inflammasome activation and IL-1β expression, the protein expression levels of IL-1β and caspase-1 in THP-1 cells were assessed by western blotting and double immunofluorescence. Protein expression was much higher in the hypoxia + 7% $CO_2$ group than in the hypoxia + 5% $CO_2$ group (caspase-1: $P < 0.01$; IL-1β: $P < 0.05$). The hypoxia + 10% $CO_2$ group had the highest levels of protein expression, compared with the hypoxia + 7% $CO_2$ (caspase-1: $P < 0.01$; IL-1β: $P < 0.01$) and hypoxia + 5% $CO_2$ groups (caspase-1: $P < 0.01$; IL-1β: $P < 0.01$). Compared with the hypoxia + 10% $CO_2$ group, protein expression was significantly suppressed by treatment with an ROS scavenger, 25 mM NAC (caspase-1: $P < 0.01$), and a pan-caspase inhibitor, 10 μM Z-YVAD-FMK (IL-1β: $P < 0.01$) (Figure 5a, b; Figure 6a, b).

## Discussion

The present study showed that 7% and 10% $CO_2$ treatment increased expression of inflammation markers in hypoxic blood; specifically, expression levels of IL-1β, TNF-α, and IL-6 were increased in hypoxic whole blood cultures. Additionally, 7% and 10% $CO_2$ treatment led to enhanced activation of NLRP3 inflammasome and elevated production of IL-1β via ROS overproduction in hypoxia-activated THP-1 cells. The model of cellular hypercapnia/hypoxemia used in the present study was originally established in our previous study.[16] In this model, 0.02% $O_2$ treatment reduced $PO_2$ levels in cell culture supernatant to approximately 60 mmHg. Treatment with 7% and 10% $CO_2$ maintained pH levels in cell culture supernatant at 7.20 to 7.30. These findings are consistent with the hypoxemia and permissive hypercapnia changes observed in patients with ARDS.[27–30]

Hypercapnia has been reported to attenuate endotoxin-induced inflammation by inhibiting activation of nuclear factor-κB.[14,15] In contrast, we found that treatment with 7% and 10% $CO_2$ enhanced the expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures. The previous research results may appear to contradict those of the present experiments. However, during hypercapnic hypoxia, there may be differences in the underlying mechanism whereby hypercapnia exerts its effects on the blood-mediated inflammatory

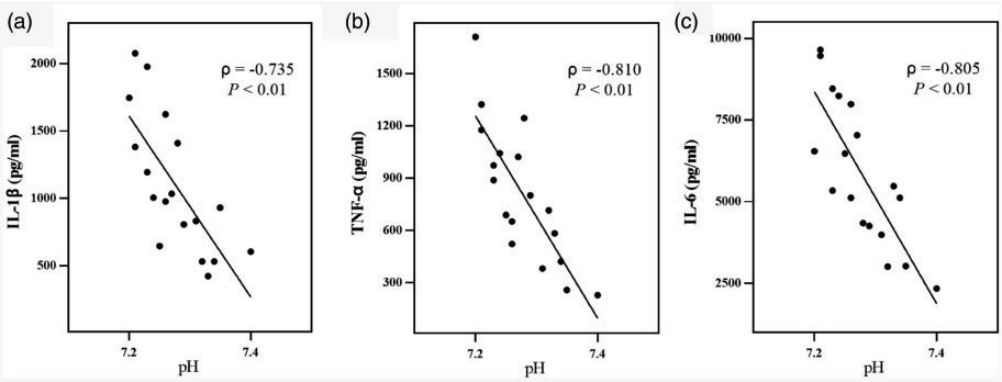


**Figure 3.** Expression levels of IL-1$\beta$ (a), TNF-$\alpha$ (b), and IL-6 (c) were negatively correlated with pH levels (n = 6 per group; analyzed with Spearman's rank correlation coefficient).
Abbreviations: IL-1$\beta$, interleukin-1 beta; TNF-$\alpha$, tumor necrosis factor-alpha; IL-6, interleukin-6

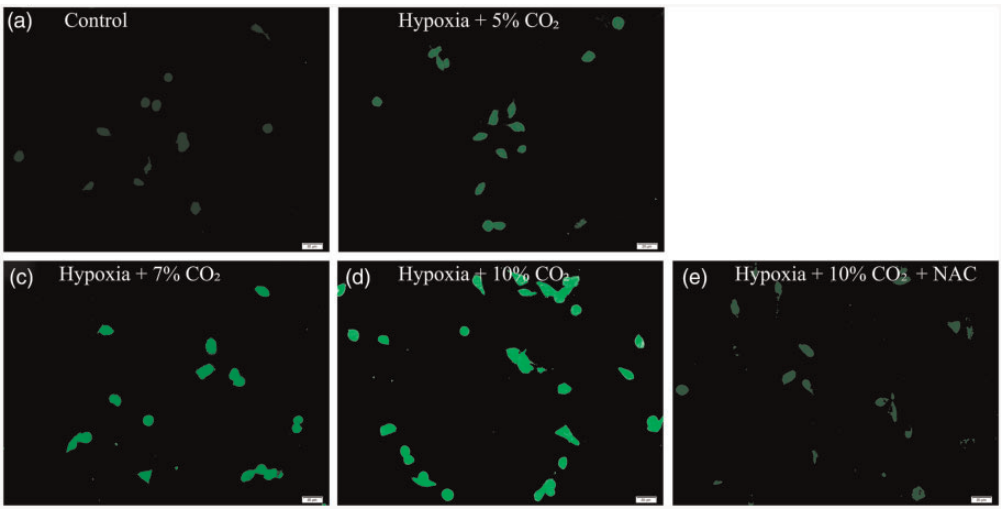


**Figure 4.** Higher levels of $CO_2$ treatment induced overproduction of ROS in hypoxic THP-1 cells (n = 4 per group). Immunofluorescence images show production of ROS (green) in hypoxic THP-1 cells. Scale bars = 20 $\mu$m. Exposures were as follows: control (20% $O_2$ + 5% $CO_2$), hypoxia + 5% $CO_2$ (5% $CO_2$ + 0.2% $O_2$), hypoxia + 7% $CO_2$ (7% $CO_2$ + 0.2% $O_2$), hypoxia + 10% $CO_2$ (10% $CO_2$ + 0.2% $O_2$), and hypoxia + 10% $CO_2$ + NAC (10% $CO_2$ + 0.2% $O_2$ + 25 mM NAC).
Abbreviations: ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine

response. Exposure to endotoxin may induce development of inflammation through the Toll-like receptor/nuclear factor-κB pathway.[31–33] Hypoxia may activate the NLRP3 inflammasome by inducing ROS overproduction, which can lead to pyroptosis and promote a cascade of inflammatory responses.[34–37] Our previous study revealed that hypercapnia-induced NLRP3 inflammasome activation in

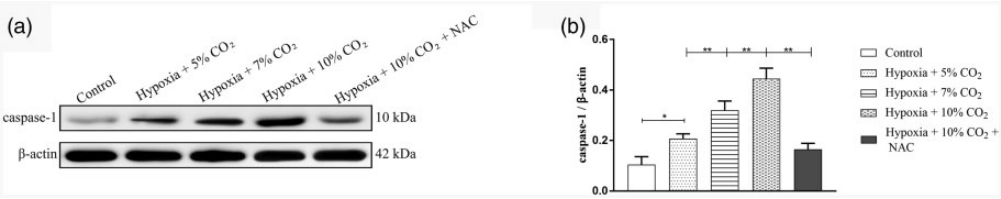


**Figure 5.** High levels of $CO_2$ enhanced activation of NLRP3 inflammasome in hypoxic THP-1 cells (n = 4 per group). (a) Immunoreactive bands of caspase-1 (10 kDa). (b) Relative density of caspase-1. $^{*}P < 0.05$, $^{**}P < 0.01$. Exposures were as follows: control (20% $O_2$ + 5% $CO_2$), hypoxia + 5% $CO_2$ (5% $CO_2$ + 0.2% $O_2$), hypoxia + 7% $CO_2$ (7% $CO_2$ + 0.2% $O_2$), hypoxia + 10% $CO_2$ (10% $CO_2$ + 0.2% $O_2$), and hypoxia + 10% $CO_2$ + NAC (10% $CO_2$ + 0.2% $O_2$ + 25 mM NAC).
Abbreviation: NLRP3, NLR family, pyrin domain-containing 3 protein; NAC, N-acetyl-L-cysteine

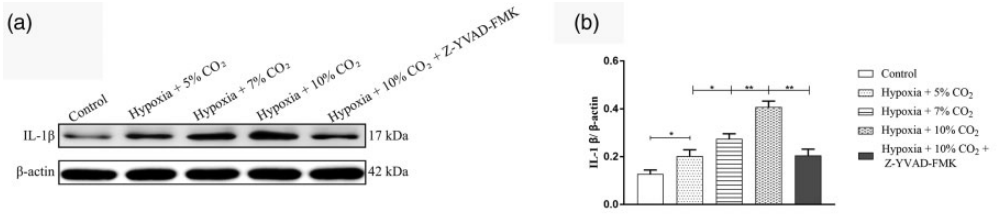


**Figure 6.** High levels of $CO_2$ increased IL-1$\beta$ expression in hypoxic THP-1 cells (n = 4 per group). (a) Immunoreactive bands of IL-1$\beta$ (17 kDa). (b) Relative density of IL-1$\beta$. $^{*}P < 0.05$, $^{**}P < 0.01$. Exposures were as follows: control (20% $O_2$ + 5% $CO_2$), hypoxia + 5% $CO_2$ (5% $CO_2$ + 0.2% $O_2$), hypoxia + 7% $CO_2$ (7% $CO_2$ + 0.2% $O_2$), hypoxia + 10% $CO_2$ (10% $CO_2$ + 0.2% $O_2$), and hypoxia + 10% $CO_2$ + Z-YVAD-FMK (10% $CO_2$ + 0.2% $O_2$ + 10 $\mu$M Z-YVAD-FMK)
Abbreviations: IL-1$\beta$, interleukin-1 beta

hypoxia-activated microglia could upregulate the expression of IL-1β in a time-dependent manner—the expression of IL-1β peaked at 24 hours of exposure to hypercapnia.[16] The previous findings are consistent with the effects on peripheral inflammation in hypoxic blood after 24 hours of treatment with 7% and 10% $CO_2$, observed in the present study.

Monocytes are reportedly the main cellular source of pro-inflammatory cytokines.[19,20] Therefore, the THP-1 human monocytic cell line was used for experiments in the present study. To explore the effects of high levels of $CO_2$ on NLRP3 inflammasome activation and IL-1β expression, the protein expression levels of IL-1β and caspase-1 in THP-1 cells were examined by western blotting. We found that the NLRP3 inflammasome could be activated in hypoxic THP-1 cells exposed to high levels of $CO_2$, as demonstrated by overproduction of ROS and upregulation of caspase-1 and IL-1β. These results suggest that high levels of $CO_2$ can contribute to onset of pyroptosis and promote a cascade of inflammatory responses through activation of the NLRP3 inflammasome in hypoxia-activated monocytic cells. This hypothesis was supported by the elevated expression levels of IL-1β, TNF-α, and IL-6 observed in hypoxic whole blood cultures in this study.

There were two limitations in this study. First, although 7% and 10% $CO_2$ treatment increased the expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures, this was an in vitro experiment. An animal experiment (such as the protocol used by Ding et al.[16]) should be performed to provide evidence regarding the effects of hypercapnia on inflammation in vivo. Second, it remains unclear how high levels of $CO_2$ treatment cause elevated expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures. Our results would be strengthened by investigation of specific pathways by which high levels of $CO_2$ regulate inflammation.

In summary, these preliminary findings suggest that treatment with high levels of $CO_2$ increases the expression levels of IL-1β, TNF-α, and IL-6 in hypoxic whole blood cultures. Permissive hypercapnia may enhance inflammation in the blood of patients with ARDS who exhibit hypoxemia.

## Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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