Basic Medical Research

Elevated extracellular CO_2 level affects the adaptive transcriptional response and survival of human peripheral blood mononuclear cells toward hypoxia and oxidative stress

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ABSTRACT

BACKGROUND High carbon dioxide (CO_2) level from indoor environments, such as classrooms and offices, might cause sick building syndrome. Excessive indoor CO_2 level increases CO_2 level in the blood, and over-accumulation of CO_2 induces an adaptive response that requires modulation of gene expression. This study aimed to investigate the adaptive transcriptional response toward hypoxia and oxidative stress in human peripheral blood mononuclear cells (PBMCs) exposed to elevated CO_2 level *in vitro* and its association with cell viability.

METHODS PBMCs were treated in 5% CO_2 and 15% CO_2, representatives a high CO_2 level condition for 24 and 48 hours. Extracellular pH (pHe) was measured with a pH meter. The levels of reactive oxygen species were determined by measuring superoxide and hydrogen peroxide with dihydroethidium and dichlorofluorescin-diacetate assay. The mRNA expression levels of hypoxia-inducible factor (HIF)-1α, HIF-2α, nuclear factor (NF)-κB, and manganese superoxide dismutase (MnSOD) were analyzed using a real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Cell survival was determined by measuring cell viability.

RESULTS pHe increased in 24 hours after 15% CO_2 treatment, and then decreased in 48 hours. Superoxide and hydrogen peroxide levels increased after the 24- and 48-hour of high CO_2 level condition. The expression levels of NF-κB, MnSOD, HIF-1α, and HIF-2α decreased in 24 hours and increased in 48 hours. The increased antioxidant mRNA expression in 48 hours showed that the PBMCs were responsive under high CO_2 conditions. Elevated CO_2 suppressed cell viability significantly in 48 hours.

CONCLUSIONS After 48 hours of high CO_2 level condition, PBMCs showed an upregulation in genes related to hypoxia and oxidative stress to overcome the effects of CO_2 elevation.

KEYWORDS elevated CO_2, extracellular pH, oxidative stress, PBMC, reactive oxygen species

Carbon dioxide (CO_2) in the atmosphere is produced by cellular metabolism as a residual product of cellular respiration.[1] Elevated CO_2 level could come from indoor environments with inadequate air ventilation, such as classrooms and offices, and could cause permanent exposure to high CO_2. Jakarta as a metropolitan city is dominated by high-rise buildings, 53.62% of which do not meet the healthy building criteria from the Environmental Protection Agency.[2] This issue causes an increased level of indoor CO_2, leading to a sick building syndrome in workers.

Excessive indoor CO_2 level of 20–50% causes hypercapnia, which is evidenced by a high CO_2 level of 7.5–20% in blood.[1] Physiologically, the CO_2 level in

human blood is less than 5%.[3] Nevertheless, Casalino-Matsuda et al[4] showed that 20% of CO_2 in blood and tissue could interfere with essential innate immune functions by decreasing phagocytosis in macrophage. Over-accumulation of CO_2 can be perceived by cells and induces an adaptive response. The adaptive response refers to the ability of cells to fight damage caused by exposure to toxic agents or stress conditions, which requires various modulation of gene expression. A previous study reported that elevated blood CO_2 level affects the transcription of many genes in bronchialepithelial cells; for instance, it upregulates lipid metabolism genes and downregulates immune response genes.[4]

An increased of CO_2 level in the blood decreases O_2 level, which causes hypoxia. Hypoxia is a condition characterized by an inadequate supply of O_2, resulting in low blood O_2 level.[5] Hypoxia-inducible factors (HIFs), known as oxygen sensors, are transcription factors that regulate gene expression in response to hypoxia.[6] HIF-1α and HIF-2α activate glucose transporter gene transcription to support an anaerobic glycolysis due to hypercapnia or hypoxia, which produces lactate. Thus, it decreases extracellular pH (pHe).[7,8]

Hypoxia also causes the accumulation of reactive oxygen species (ROS), which induces oxidative stress. Oxidative stress is a state of imbalance between prooxidants and antioxidants. In response to oxidative stress, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor regulates the gene expression of many antioxidants. A major mitochondrial antioxidant that is regulated by NF-κB is manganese superoxide dismutase (MnSOD), which converts toxic superoxide anion to less toxic hydrogen peroxide-free radicals.[9]

Until now, *in vitro* studies on the adaptive mechanism of cell responses toward elevated blood CO_2 are still limited. Therefore, this study aimed to elucidate the adaptive transcriptional response toward hypoxia and oxidative stress in human peripheral blood mononuclear cells (PBMCs) exposed to elevated CO_2 level *in vitro* and its association with cell viability. PBMCs, which include monocytes, macrophages, and lymphocytes, are parts of the immune system.[10] The present study used PBMCs because these cells play essential roles in the immune responses and may represent other cellular adaptive responses toward systemic conditions. Understanding the cellular adaptive responses

toward increased blood CO_2 level is important for early detection and management of inadequate indoor air quality.

METHODS

This study enrolled 20 students attending the Faculty of Medicine Universitas Indonesia. They have lived in Jakarta for 5 years, male, healthy, and aged 18–21 years. The exclusion criteria were smokers, athletes, drug abusers, and those with chronic disorders. All participants had given their consent prior to study participation. This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No: 830/UN2.F1/ETIK/2017).

A volume of 3 ml venous blood sample from each subject was collected into the EDTA vacutainers. PBMC isolation was performed using Histopaque® (Sigma-Aldrich, USA) in accordance with the manufacturer's protocol. The PBMCs were resuspended in Advanced Roswell Park Memorial Institute 1640 medium (Gibco®, Thermo Fisher Scientific Inc., USA) supplemented with 10% fetal bovine serum (Gibco®, Thermo Fisher Scientific Inc.), 1% penicillin-streptomycin (Gibco®, Thermo Fisher Scientific Inc.), 1% amphotericin B (Gibco®, Thermo Fisher Scientific Inc.), and 1% phytohaemagglutinin (Gibco®, Thermo Fisher Scientific Inc.). Then, their pH was adjusted to 7.4 by adding 0.01 M of hydrochloric acid (Merck KGaA, Germany).

A total of 2.5×10^5 PBMCs were seeded in a 24-well plate and divided into two groups: first group in standard cell culture condition of CO_2 level (5%) and second group was incubated in elevated 15% CO_2 level representatives as high CO_2 level condition. After 24 and 48 hours, the PBMCs were harvested and centrifuged at $200 \times g$ for 10 min. The supernatant was transferred into a new tube for pHe measurement using a pH meter (Milwaukee Instruments, Inc., USA). The pellet was resuspended with 1 ml of medium, and the viable cells were determined with a trypan blue exclusion method using a hemocytometer.

The superoxide level was measured using dihydroethidium (DHE) assay (Invitrogen™ Molecular Probes™, Thermo Fisher Scientific Inc., USA), whereas the hydrogen peroxide level was determined using 2',7'-dichlorofluorescin-diacetate (DCFH-DA) assay (Invitrogen™ Molecular Probes™, Thermo Fisher Scientific Inc.). PBMCs were washed twice with sterile

Table 1. Primer sequences and annealing temperatures

Gene	Primer sequences	Annealing temperature (°C)
18sRNA	(Forward) 5'-AAACGGCTACCACATCCAAG-3'	60
	(Reverse) 5-CCTCCAATGGATCCTCGTTA-3'	
NF-κB	(Forward) 5'-CGCTTAGGAGGGAGAGCCCA-3'	55
	(Reverse) 5'-TGGGCCATCTGTTGGCAGTG-3'	
MnSOD	(Forward) 5'-CTACGTGAACAACCTGAACGTCACC-3'	60
	(Reverse) 5'-TGAACTTCAGTGCAGGCTGAAGAG-3'	
HIF-1α	(Forward) 5'-GGCGCGAACGACAAGAAAAAG-3'	54.6
	(Reverse) 5'-AGTGGCAACTGATGAGCAAG-3'	
HIF-2α	(Forward) 5'-ATAGCAGTGGCAAGGGGGCT-3'	60
	(Reverse) 5'-TCAGGGCTATTGGGCGTGGA-3'	

HIF=hypoxia-inducible factor; MnSOD=manganese superoxide dismutase; NF-κB=nuclear factor kappa-light-chain-enhancer of activated B cells

phosphate buffer saline (PBS) (Gibco®, Thermo Fisher Scientific Inc.), suspended in 500 μl of PBS, and then incubated with 20 μM DHE or 20 μM DCFH-DA for 30 min at 37°C in the dark. Fluorescence intensity was measured using a fluorometer (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific Inc., USA) with excitation and emission wavelengths of 480 and 585 nm for DHE and 485 and 530 nm for DCFH-DA, respectively. Absorbance reflected the concentrations of superoxide and peroxide produced from each treatment. Results were shown by dividing the absorbance with the number of cell viability after treatment.

Total RNA was isolated using TriPure Isolation Reagent (Roche Applied Science, Switzerland) in accordance with the manufacturer's instructions. Total RNA concentration was quantified using spectrophotometry at 260 nm (Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific Inc.). The level of mRNA expression was quantified using a real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) (Applied Biosystems™ 7500 Fast Real-Time PCR System, USA) using SensiFast™ SYBR®

No-ROX One-Step Kit (Bioline Reagents LTD., UK) in accordance with the manufacturer's instructions. The primers and annealing temperatures are described in Table 1. All reactions were performed in triplicate. The relative expression levels were calculated using Livak's formula[11] and normalized (Δ cycle threshold [Ct] of treatment groups was substracted to ΔCt of control group) to each control group. Data are presented as the mean (standard error of the mean).

All values in the boxplot are reported as median (minimum–maximum). Variables were assessed for normality by Shapiro–Wilk test. Significant differences were set at $p < 0.05$ and $p < 0.01$, determined using independent t-test in comparison with the 24-hour treatment and the control (5% CO_2). These tests were performed using SPSS version 20 (IBM Corp., USA).

RESULTS

To examine the effect of elevated CO_2 in cell environment, pHe was measured immediately after the 24- and 48-hour high CO_2 treatments. Treatment duration affected pHe, as shown in Table 2. The pHe

Table 2. pHe in sample after 24 and 48 hours of treatment

	Before incubation	After 24 hours	After 48 hours	Δ 24 hours*	Δ 48 hours*	p
Standard CO₂ conditions (5%)	7.40 (0.00)	7.58 (0.22)	7.20 (0.23)	0.19 (0.05)	−0.20 (0.05)	**<0.001**
High CO₂ conditions (15%)	7.40 (0.00)	7.53 (0.182)	7.14 (0.184)	0.14 (0.04)	−0.26 (0.04)	**<0.001**

pHe=extracellular pH
*Mann–Whitney t-test. ΔpHe is the difference between pH after and before incubation
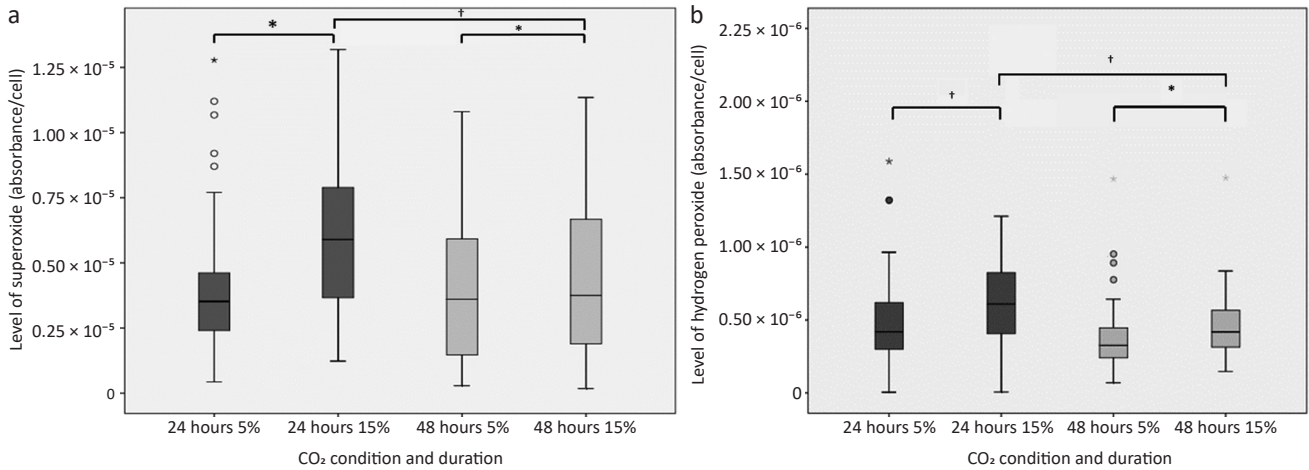
Figure 1. ROS levels after CO_2 treatment. (a) Superoxide level after incubation with 15% CO_2 for 24 and 48 hours were increased from 0.37 to 0.43 × 10^{-5} ($p<0.01$) with 25th-75th percentils, 0.19-0.77 x 10^{-5}. Superoxide level was presented as absorbance of fluorescence intensity of DHE in PBMCs (absorbance per cell); (b) levels of hydrogen peroxide after treatment with 5% and 15% CO_2 for 24 and 48 hours were 0.352 and 0.59 × 10^{-6} ($p<0.05$) (25th-75th percentils, 0.1-1.3 × 10^{-6}). Hydrogen peroxide was determined using DCFH-DA assay. Data are presented as mean (SD). Statistical analysis was performed using Mann–Whitney test compared with the control (cell under normal 5% CO_2 conditions). DCFH-DA=2',7'-dichlorofluorescin-diacetate; DHE=dihydroethidium; PBMCs=peripheral blood mononuclear cells; ROS=reactive oxygen species; SD=standard deviation
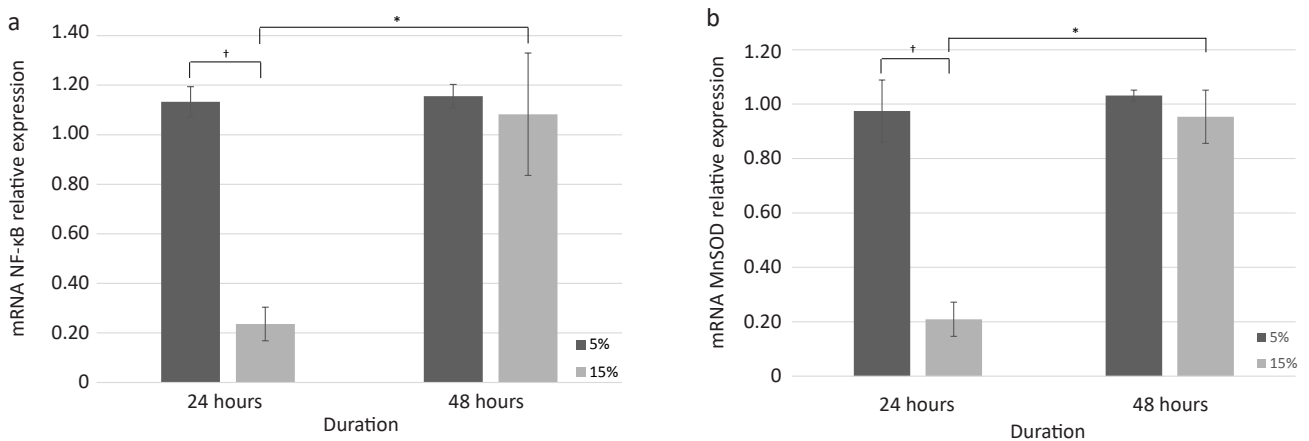*$p<0.05$; †$p<0.01$ compared with the control

Figure 2. Suppression of NF-κB affected MnSOD mRNA relative expression. (a) Levels of NF-κB mRNA relative expression were not significantly suppressed in high CO_2 conditions after 24 hours compared with 48 hours; (b) MnSOD expression decreased in high CO_2 conditions after 24 hours and increased after 48 hours compared with standard CO_2 conditions. Data of treated cells were normalized to control (5% CO_2). Data are presented in mean (SE). MnSOD=manganese superoxide dismutase; NF-κB=nuclear factor kappa-light-chain-enhancer of activated B cells; SE=standard error of the mean
*$p<0.05$; †$p<0.01$ using Mann–Whitney independent t-test

in the high CO_2 conditions was higher than that in the standard CO_2 conditions in 24 hours. After the 48-hour treatment, pHe significantly decreased (ΔpHe = −0.26 [0.04]) compared with the PBMCs in standard CO_2 conditions (ΔpHe = −0.20 [0.05]). These results showed that the PBMCs produced metabolic products, such as H^+, which decreased the pHe after treatment (Table 2).

DHE and DCFH-DA assays were performed to determine the effect of elevated CO_2 level on human PBMCs. Figure 1a shows that the level of superoxide significantly increased after 24 hours in

high CO_2 conditions compared with standard CO_2 conditions. Moreover, the hydrogen peroxide level was significantly higher in high CO_2 conditions after 24 hours, as shown in Figure 1b. Both superoxide and hydrogen peroxide decreased in high CO_2 conditions after 48 hours. This indicates that PBMCs had cell adaptive response in improving oxidative stress status after 24 hours exposed to high CO_2. Furthermore, the mRNA relative expression of antioxidant agents was analyzed to determine the defense mechanism of PBMCs exposed to elevated CO_2.
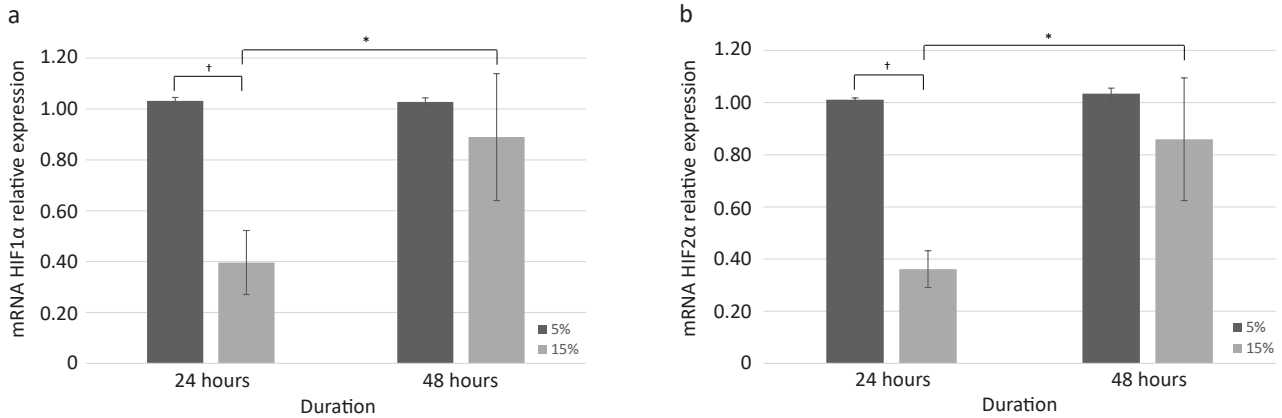
Figure 3. Relative expression of HIF-1α (a) and HIF-2α (b) mRNA in PBMCs after incubation with 15% CO_2 for 24 and 48 hours. mRNA expression was measured using qRT-PCR and calculated using Livak formula. Data on treated cells were normalized to control. Data are presented in mean (SE). Statistical analysis was perfomed using Mann–Whitney independent *t*-test. HIF=hypoxia-inducible factor; PBMCs=peripheral blood mononuclear cells; qRT-PCR=real time reverse transcriptase-polymerase chain reaction; SE=standard error of the mean
*$p<0.05$; †$p<0.01$ compared with the control

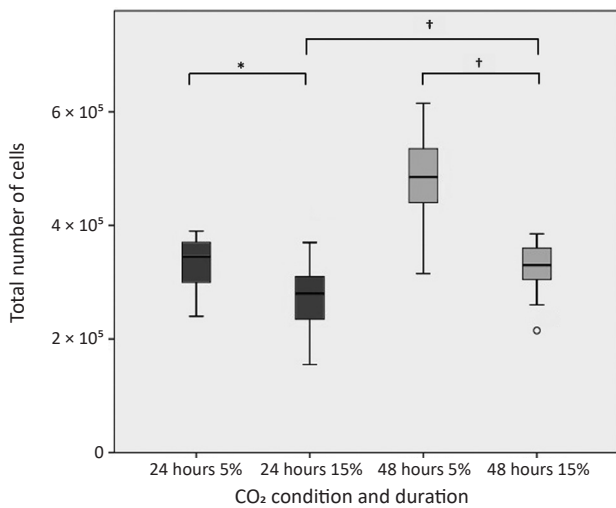**Figure 4.** Viability of peripheral blood mononuclear cells (PBMCs) after treatment with 15% CO_2 in 24- and 48-hour incubation. Cell viability was measured using trypan blue exclusion assay. Viability decreased in high CO_2 conditions compared with standard CO_2 conditions
*$p<0.05$; †$p<0.01$ using Mann–Whitney *t*-test

In this study, the levels of NF-κB mRNA relative expression were not significantly suppressed in high CO_2 conditions after 24 hours (0.24-fold; $p<0.01$) compared with after 48 hours (1.08-fold) (Figure 2a). *NF-κB* suppression also affected *MnSOD* mRNA expression. *MnSOD* mRNA relative expression depleted to 0.21-fold ($p<0.01$) when incubated for 24 hours. After 48 hours in high CO_2 conditions, *MnSOD* mRNA expression increased to 0.95-fold, as shown in Figure 2b.

The mRNA expression of HIF-1α and HIF-2α was also examined to assess the effect of O_2 concentration

after treatment. Similar results were observed in NF-κB and MnSOD expression. After treatment with 15% CO_2, no significant difference was observed in HIF-1α expression after 24 and 48 hours incubation compared with standard CO_2 conditions, as shown in Figure 3a. The results were shown by a significant decrease (0.40-fold) of PBMCs after 24 hours in high CO_2 conditions compared with standard CO_2 ($p<0.01$). The relative expression of *HIF-1α* increased to 0.89-fold after 48 hours in high CO_2 conditions. Meanwhile, the *HIF-2α* mRNA expression also reduced after 24 hours in high CO_2 conditions (0.36-fold; $p<0.01$) and increased after 48 hours in high CO_2 condition (0.86-fold), as shown in Figure 3b. These results showed that the expression levels of NF-κB, MnSOD, HIF-1α, and HIF-2α in 24 hours decreased and started to increase in 48 hours, although the expression was only slightly higher in standard CO_2 conditions. The enhancement of antioxidant mRNA relative expression in the high CO_2 conditions after 48 hours showed that the PBMCs have adaptive transcriptional responses under high CO_2 conditions.

The impact of elevated CO_2 in PBMCs on cell viability was analyzed using trypan blue exclusion assay through Shapiro–Wilk test. The total cell in 5% CO_2 in the 24-hour treatment was significantly higher (median = 350,000 cells/well) than that in the 15% CO_2 (median = 275,000 cells/well) ($p<0.05$). Furthermore, the median of viable cells was higher in standard CO_2 than high CO_2 conditions (485,000 cells/well *versus* 335,000 cells/well, $p<0.01$). Cells continued to multiply

along with the length of time exposure, but the result showed that high CO_2 decreased the viability after 24 hours and led to significant decrease after 48 hours ($p<0.01$) compared with standard CO_2 (Figure 4).

DISCUSSION

The increased level of CO_2 in the blood is enhanced by excessive indoor CO_2 level. In this experiment, the exposure of the PBMC culture medium to high CO_2 level led to acidification. In general, pHe depends on the balance of CO_2 level and bicarbonate (HCO_3^-) as a pH buffer. Alteration of CO_2 level would make the cells adapt by changing the pH of the medium. Our study demonstrated that the pHe of the PBMCs decreased after treatment with 15% CO_2, independent to the duration of exposure. We suggest that high CO_2 level is hydrated to H^+ and HCO_3^- and facilitated the cells to release H^+, leading to low pHe in PBMCs.[12] Acidification in PBMC environment can induce the expression of carbonic anhydrase (CA), which plays an important role in regulating intracellular and pHe. CA has a capacity to maintain the growth and survival of the cells by maintaining intracellular pH to ~7.4, which is an optimum condition.[13]

Acidic microenvironment can also affect glycolysis in cancer. Warburg effect, the phenomenon of aerobic glycolysis, occurs when the availability of O_2 in the environment is sufficient to create large amounts of lactic acid as the final product.[13] Hence, the production of lactate due to change in an acidic environment can reduce oxygen levels, leading to hypoxia, which upregulates the stress-induced chaperone heat shock protein 90 through prolyl hydroxylase 2 and Von-Hipple-Lindau-independent regulation.[14] Therefore, our results showed that high CO_2 level could indirectly induce hypoxia.

The acidification that occurs in PBMCs due to high CO_2 level exposure can be related to ROS production. Acidification can increase superoxide and hydrogen peroxide production by increasing the pH gradient between the intermembrane space of mitochondria and mitochondrial matrix.[15] A decreased O_2 level due to elevated CO_2 upregulates xanthine dehydrogenase, which is converted to xanthine oxidase during post-translational modification, and leads to the production of hydrogen peroxide.[16] In the present study, the levels of superoxide and hydrogen peroxide significantly increased in high CO_2 conditions after 24

hours compared with standard CO_2 conditions. The enhancement was not significant after 48 hours in high CO_2 conditions. These results may be attributed to the activities of catalase and glutathione peroxidase.

The mechanisms controlling the induction of genes by oxidative stress have been intensively investigated. Transcription factors, such as NF-κB, are stimulated by ROS. The antioxidant-responsive element sequence plays an important role in gene promoters induced by oxidative stress. One of these proteins has recently been shown to be suppressed by oxidative stress. Thus, ROS can interfere with gene expression at the transcriptional level.[17] Our study indicated that the increase in ROS levels was in accordance with the suppression of NF-κB mRNA expression.

High CO_2 might suppress the expression of genes associated with innate immunity and inflammation, which are linked to the NF-κB pathway as the main regulator. NF-κB regulates the expression of genes involved in cell growth, differentiation, development, and apoptosis. NF-κB regulation has two pathways. The canonical pathway begins with the stimulation of proinflammatory receptors, such as Toll-like receptor, tumor necrosis factor receptor, and cytokine receptors. The canonical pathway controls the expression of genes that protect the cells from oxidative stress, such as MnSOD. By contrast, the noncanonical pathway is initiated by NF-κB-inducing kinase (NIK), which elicits the expression of ROS-generating genes and supports anti-inflammatory or immunosuppressive effects.[10,18] Based on the results, the decreased of NF-κB mRNA relative expression after treatment is probably because of the noncanonical pathway activation. The noncanonical pathway liberates and activates the NF-κB molecules in the cytosol. Thus, the cells do not have to synthesize more NF-κB because the proteins have already been activated in the cytosol. However, the increase in NF-κB expression in high CO_2 conditions after 48 hours showed that ROS influence NF-κB as they oxidize cysteine, thereby activating NIK that initiates the noncanonical pathway.

In the presence of ROS, NF-κB would modulate the expression of target genes (e.g., *MnSOD*) that promote survival. NF-κB during high CO_2 level has pro-oxidant properties that further suppress the expression of *MnSOD*. *MnSOD* transcription is part of the canonical pathway. This study showed that downregulating the

mRNA expression of MnSOD may break down MnSOD DNA strand and cause more mitochondrial damage. The activity of microRNA-183, which can inhibit the expression of isocitrate dehydrogenase 2, causes an impaired function of the mitochondria.[19]

The immunosuppressive effect of high CO_2 does not directly affect HIFs. The synthesis of HIF-1α can be regulated by oxygen-independent pathways involving signaling molecules, such as NF-κB.[20] High CO_2 level suppresses HIF-1α levels and activities in response to hypoxia.[15] The acute episode of ROS production can also stimulate the synthesis of HIF-1α through the induction of the phosphatidylinositol 3-kinase/protein kinase B and extracellular-signal-regulated kinase signaling pathways, leading to the rapid accumulation of the protein.[20] The expression of HIF-1α mRNA was lower after 24 hours than 48 hours in high CO_2 conditions. These results suggest that the HIF-1α protein concentration reaches its peak at some point prior to 24 hours, and this level of HIF-1α induces the negative feedback mechanism against the HIF-1α mRNA expression to become remarkably decreased in this acute period.

Unlike HIF-1, HIF-2α plays a larger role in tissue revascularization along with regenerative processes.[18] This condition affects the degradation of HIF-2α protein but not its mRNA. Although it was still lower than in standard CO_2 conditions, the increased expression level after 48 hours in HIF-1α and HIF-2α suggests the possibility of a compensatory mechanism that can increase the expression of HIF-1 after acute exposure to CO_2. However, the longer oxygen deprivation might not only inhibit protein degradation but also affect the upstream regulation of *HIF* genes.

The above discussion indicates that the impairment of mitochondria is the contributor to decreased cell viability. An acidic extracellular environment can decrease cell proliferation by disrupting the electron transport that would release free radicals outside the mitochondria, causing free radical leakage. The leakage affects the cell cycle which inhibits cell proliferation.[21] The increased level of ROS leads to DNA damage. When DNA damage occurs, the cell cycle regulation system would slow down, and apoptosis might occur,[19] as evidenced by the significant depletion in cell viability after 24 hours in high CO_2 conditions. After 48 hours in high CO_2 conditions, cell viability increased, indicating that the cells can compensate the apoptosis by increasing the rate of cell proliferation.

One of the limitations of this study is the short duration of exposure. For further research, we propose to prolong the CO_2 exposure duration, analyze CA9 mRNA expression to clarify its role in pH balancing, and investigate the immune response of PBMCs. Even though the number of viable cells decreased after 48 hours in high CO_2 conditions, the hydrogen peroxide level and mRNA expression levels still increased. In conclusion, PBMCs elicit a response to overcome the elevation of CO_2 level after 48 hours in high CO_2 conditions.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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