Inflammatory and oxidative stress-associated factors in chronic intermittent hypoxia in Chinese patients, rats, lymphocytes and endotheliocytes

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Received December 1, 2016; Accepted July 5, 2017

DOI: 10.3892/mmr.2017.7632

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Key words: inflammation, oxidative stress, obstructive sleep apnea hypopnea syndrome, chronic intermittent hypoxia

Abstract. In order to investigate the association between inflammatory and oxidative stress (OS)-associated factors in chronic intermittent hypoxia (CIH), 238 CIH patients and 156 healthy volunteers were included. CIH rat and lymphocytes were used as experimental models. Interleukin (IL)-6, tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), nitric oxide (NO) and nitric oxide synthase (NOS) were analyzed. Patients with CIH were older, with hypertension, increased heart rate (HR) and body mass index (BMI), and there were more males than females. Those with a history of smoking or type 2 diabetes (T2DM) history exhibited an increased risk of CIH. Serum IL-6, TNF-α and CRP in patients with CIH were increased, while NO and NOS were decreased. Hakka patients exhibited increased BMI measurements and NO expression, and decreased systolic arterial pressure, IL-6 and TNF-α compared with non-Hakka patients. Rats with CIH exhibited hypertension and stable weight, less activity and decreased appetite, increased HR and serum IL-6, TNF-α and CRP, and decreased NO and NOS. IL-6, TNF-α, CRP, NO and induced-NOS (iNOS) were increased in the lymphocytes of CIH rats compared with healthy ones. In rat endotheliocytes induced by CIH, IL-6, TNF-α, CRP and iNOS increased, while NO and endothelial-NOS (eNOS) decreased. In the supernatant of co-cultured lymphocytes and endotheliocytes, IL-6, TNF-α and CRP increased, although NO and NOS decreased. In conclusion, IL-6, TNF-α and CRP, and decreasing NO and NOS are biomarkers of CIH, which could be targets in diagnosis, treatment and prevention of CIH.

Introduction

The most common type of chronic intermittent hypoxia (CIH) is obstructive sleep apnea hypopnea syndrome (OSAHS), which is characterized by repeated upper airway obstruction and frequently-interrupted breathing during sleep; the condition is potentially life-threatening (1). By enhancing oxidative stress (OS), inflammation and sympathetic nerve discharge, CIH, in addition to sleep structure disorders, may stimulate a series of alterations in the molecular biology of the patient (2).

CIH is characterized by a low oxygen period, distinct from a normal oxygen period (11). However, CIH, in addition to continuous hypoxia, may induce inflammation. The underlying mechanisms of OSAHS-induced hypertension remain to be elucidated. It has previously been demonstrated that the circulating levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α and C-reactive protein (CRP) are elevated in patients with OSAHS (12-15), indicating that CIH may be associated with systemic inflammation. In the initiation and progression of hypertension, endothelial dysfunction occurs early, in response to the inflammatory cytokines (16). This process may result in an increase in intercellular adhesion molecules, leading to the promotion of leukocyte adherence to the endothelium, in addition to extravasation (16). Hypertension may subsequently develop.
Additionally, the hypoxia-reoxygenation cycles during CIH may induce the production of reactive oxygen species (ROS) and OS (17). Following CIH exposure, endothelin 1 and angiotensin II have been observed to increase due to ROS-mediation, which may enhance chemosensitivitiy in carotid bodies (18,19), while nitric oxide synthase (NOS) may inhibit chemoreceptor function (20), including endothelial NOS (eNOS) and neuronal NOS (nNOS).

There are numerous individuals belonging to the Hakka population in Guangdong Province (China), and these individuals are generally very careful about their health. Certain common customs within this population, for example, stewing soup for >2 h, making wine, plum liqueur or snake wine and cooking with less salt or oil, are claimed to be beneficial to health and in reducing diseases. Wine has been confirmed to soften the vasculature and prevent cardiovascular diseases (21), and slow cooking soup has been anecdotally claimed to maintain beauty and good health. However, the incidence of CIH within the Hakka population has not previously been investigated.

Therefore, the present study aimed to investigate the association between inflammation, OS, CIH and hypertension by comparing the levels of IL-6, TNF-α, CRP, nitric oxide (NO) and NOS in Hakka patients from Huizhou City with non-Hakka patients. Additionally, CIH rat models were established and the 5 factors were detected in isolated lymphocytes and co-cultured endotheliocytes.

**Materials and methods**

**Patients.** A total of 238 patients, including 106 Hakka patients and 132 non-Hakka patients, from Huizhou (China), were diagnosed with CIH combined with hypertension in Nanfang Hospital (Guangzhou, China) between March 2013 and October 2016. A total of 156 volunteers (non-Hakka; from Huizhou) were recruited as healthy controls during the same time periods as the patients. Clinical data including age, sex, weight, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), heart rate (HR), smoking and type 2 diabetes (T2DM) history were obtained for analysis. Blood samples were obtained at 9:00 a.m. at the initial clinic visit and stored at -28°C.

Informed consent was obtained from each patient and volunteer, and the present study was approved by the Medical Ethics Committees of Nanfang Hospital (Guangzhou, China) and Renmin Hospital of Wuhan University (Wuhan, China).

**Animals.** A total of 10 male Wistar rats (age, 60-70 days; weight, 191.42±14.05 g) were obtained from Qingdao KangDa Biotechnology Co., Ltd. (Qingdao, China) and housed individually in polycarbonate cages with wire lids, under the conditions of 12-h light/dark cycle, 22-25°C and 50-70% humidity. Standard laboratory diet and water were supplied freely. The animal welfare and experimental protocols were approved by the Animal Research Ethics Committee of Renmin Hospital of Wuhan University. The experiments were performed in accordance with the National Institutes of Health Principles of Laboratory Animal Care (NIP Publication 85-23, revised 1996), the European Guidelines for the Protection of Animals used for Scientific Purposes (European directive 2010/63/EU) and Portuguese Law no. 113/2013.

CIH models. The 10 rats were randomly divided into 2 groups, control and CIH. Rats in the CIH group were placed in a dynamic O₂/N₂ controller (OxyCyclerA844A-chamber; BioSpherix, Ltd., New York, NY, USA) and exposed to 5 min cycles of 90 sec hypoxia (5% O₂) and 210 sec normoxia (21% O₂), 8 h/day for 3 weeks during the light hours. Rats in the control group were exposed to the same environment but with normoxia (21% O₂) maintained 24 h/day for 3 weeks. The MouseOX pulse oximetry system (Starr Life Sciences Corp., Oakmont, PA, USA) was used to measure the arterial oxygen saturation (SaO₂) as ~70%, according to the specifications of the instrument.

**Hemodynamic analysis.** SAP, DAP, MAP and HR in human subjects were measured according to the clinical protocol ‘Essential hypertension for primary healthcare level’ (22). Hemodynamic analysis in rats was performed using Mouse and Rat Tail Cuff Blood Pressure system and analyzed using the equipped software (3R22931; IITC Life Science, Inc., Woodland Hills, CA, USA), according to the manufacturer's specifications, one day prior to model establishment and at the 7th, 14th and 21st days of experimentation.

**Serum IL-6, TNF-α, CRP, NO and NOS detection.** For patients, blood samples were concentrated at 3,000 x g at 4°C for 10 min and serum was separated. For rats, weights were measured and blood was collected at 9:00 a.m., one day prior to model establishment and on the 7th, 14th and 21st day of experiments. Blood samples were centrifuged at 3,000 x g for 10 min at 4°C to separate the serum. ELISA kits for Serum IL-6 (CHC1263, Thermo Fisher Scientific, Inc., Waltham, MA, USA), TNF-α (RAB0340) and CRP (CYT298) (both from Sigma-Aldrich; Merck KGaA) were used. NO and NOS levels were determined using Total Nitric Oxide Assay and Nitric Oxide Synthase Assay kits (Beyotime Institute of Biotechnology, Haimen, China), respectively.

**Lymphocyte isolation.** The lymphocytes of rats in the two groups were isolated using Ficoll-Histopaque (Sigma-Aldrich; Merck KGaA) double density gradient centrifugation. A total of 3 ml Histopaque 1119 was added to a 15 ml tube, and 3 ml of 3 ml Histopaque 1083 was added to the control group were exposed to the same environment but with normoxia (21% O₂) maintained 24 h/day for 3 weeks. The MouseOX pulse oximetry system (Starr Life Sciences Corp., Oakmont, PA, USA) was used to measure the arterial oxygen saturation (SaO₂) as ~70%, according to the specifications of the instrument.

The 10 rats were randomly divided into 2 groups, control and CIH. Rats in the CIH group were placed in a dynamic O₂/N₂ controller (OxyCyclerA844A-chamber; BioSpherix, Ltd., New York, NY, USA) and exposed to 5 min cycles of 90 sec hypoxia (5% O₂) and 210 sec normoxia (21% O₂), 8 h/day for 3 weeks during the light hours. Rats in the control group were exposed to the same environment but with normoxia (21% O₂) maintained 24 h/day for 3 weeks. The MouseOX pulse oximetry system (Starr Life Sciences Corp., Oakmont, PA, USA) was used to measure the arterial oxygen saturation (SaO₂) as ~70%, according to the specifications of the instrument.

**Detection of apoptosis rate and IL-6, TNF-α, CRP, NO and iNOS levels in lymphocytes.** Isolated lymphocytes in the logarithmic growth phase were used for the following experiments (1x10⁵ cells/ml). A propidium iodide staining kit (Beyotime
Institute of Biotechnology) was used to detect apoptosis in rat lymphocytes, according to the manufacturer's protocol, and the results were observed under a fluorescence microscope at 535 nm. Western blotting was used to detect IL-6, TNF-α, CRP and iNOS levels in lymphocytes. Radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) was added to the lymphocytes according to the manufacturer's protocol. The cells were incubated on ice for 10 min and centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was separated and 5X loading buffer (Beyotime Institute of Biotechnology) was added. The total protein concentration was determined using a bicinchoninic acid protein quantitation kit (Beyotime Institute of Biotechnology). A total of 40 μg protein/lane was separated by SDS-PAGE with a 5% stacking gel and a 10% separating gel. The proteins were transferred to polyvinylidene fluoride membranes (Merck KGaA). Rabbit anti-IL-6 (21865-1-AP, 1:2,000), rabbit anti-TNF-α (17590-1-AP, 1:2,000), rabbit anti-CRP (13432-1-AP, 1:2,000), rabbit anti-iNOS (18985-1-AP, 1:2,000), mouse anti-β-actin (60008-1-AP, 1:10,000) (all from Wuhan Sanying Biotechnology, Wuhan, China) were used to incubate at 4°C overnight. The membranes were then incubated with goat anti-mouse IgG HRP (223-005-024) or goat anti-rabbit secondary antibodies (323-005-024, 1:3,000) (both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 25°C for 1 h. Enhanced chemiluminescence (WBKLS0500; Merck KGaA) substrate was used to visualize proteins. Semi-quantification was performed using ImageJ 1.48 (National Institutes of Health, Bethesda, MD, USA). NO detection was performed using the Total Nitric Oxide Assay kit.

**Results**

**Clinical data and hemodynamic analysis in Hakka patients.** Comparisons between Hakka and non-Hakka patients with CIH combined with hypertension and healthy volunteers are presented in Table I and Fig. 1, including age, sex, body mass index (BMI), smoking and T2DM history, hemodynamic changes (SAP, DAP, MAP and HR), and serum markers (IL-6, TNF-α, CRP, NO and NOS). The odds ratio and r (correlation coefficient) values are representative of the entire cohort.

The patients with CIH combined with hypertension were older (r=0.536), and more of them were males (r=0.681). The patients exhibited increased BMI (r=0.710) compared with the healthy volunteers, while the BMI of non-Hakka patients was significantly decreased compared with Hakka patients (P<0.05). More patients had a history of smoking (r=0.611) and T2DM (r=0.692) compared with the healthy volunteers.

According to the results of the hemodynamic analysis, the patients exhibited increased SAP (r=0.542), DAP (r=0.602), MAP (r=0.575) and HR (r=0.326) compared with the healthy volunteers. However, the SAP of Hakka patients was significantly decreased compared with that of non-Hakka patients (P<0.05).

The inflammatory factors, IL-6 (r=0.787), TNF-α (r=0.619) and CRP (r=0.328), were increased in the patients compared with the healthy volunteers, while NO (r=-0.206) and NOS (r=-0.443) were decreased. The IL-6 and TNF-α levels were increased in non-Hakka patients compared with Hakka patients, while NO levels were significantly decreased (P<0.05).

**Rat weight and hemodynamic alterations.** The weight changes and comparisons between CIH and healthy rats are presented in Table II and Fig. 2A. Prior to the experiments, the average weight of the CIH rats was not significantly different from that of the control group. During the experiments, the average weight of the CIH and healthy rats increased gradually. However, the CIH rats exhibited decreased activity and appetite, resulting in the average CIH rat weight being significantly decreased compared with that of the healthy rats on the 7th, 14th and 21st days (P<0.05).

The alterations in SAP, DAP, MAP and HR in CIH and healthy rats are presented in Table III and Fig. 2B-E. Prior to experiments, there was no significant difference in SAP, DAP, MAP or HR between CIH rats and healthy rats. During the experiments, the average SAP, DAP, MAP and HR of the CIH rats increased, gradually and significantly (P<0.05). Compared
with the control, SAP, DAP, MAP and HR in the CIH rats on the 7th, 14th and 21st days were significantly increased (P<0.05).

**Serum IL-6, TNF-α, CRP, NO and NOS alterations in rats.** Serum IL-6, TNF-α, CRP, NO and NOS in CIH and healthy rats are presented in Table IV and Fig. 3. Prior to experiments, there was no significant difference in serum IL-6, TNF-α, CRP, NO or NOS between CIH rats and healthy rats. During the experiments, serum IL-6, TNF-α and CRP in CIH rats increased, while NO and NOS decreased, gradually and significantly (P<0.05). Compared with the control, serum IL-6, TNF-α and CRP in CIH rats were significantly increased, while NO and NOS were significantly decreased, on the 7th, 14th and 21st days (P<0.05).

**Correlation between IL-6, TNF-α, CRP, NO and NOS, and CIH.** The IL-6, TNF-α, CRP, NO and NOS levels...
were correlated with CIH, including SAP, DAP, MAP and HR, which are presented as the correlation coefficient (r) in Table V. IL-6 was positively correlated with SAP (r=0.572), DAP (r=0.439), MAP (r=0.640) and HR (r=0.530),
in addition to TNF-α with SAP (r= 0.673), DAP (r=0.519), MAP (r=0.436) and HR (r=0.448), and CRP with SAP (r=0.397), DAP (r=0.418), MAP (r=0.607) and HR (r=0.528); NO and NOS were negatively correlated with SAP (r=-0.568 and r=-0.473), DAP (r=-0.372 and r=-0.460), MAP (r=-0.406 and r=-0.524) and HR (r=-0.630 and r=-0.581).

**Apoptosis rate, IL-6, TNF-α, CRP, NO and iNOS in rat lymphocytes.** Apoptosis rate, IL-6, TNF-α, CRP, NO and iNOS in CIH and control rats are presented in Table VI and Figs. 4 and 5.

The apoptosis rate in CIH rats was significantly decreased compared with healthy rats (P<0.05; Fig. 4). The levels of IL-6, TNF-α, CRP, NO and iNOS were significantly increased in CIH rats compared with healthy rats (P<0.05; Fig. 5).

**IL-6, TNF-α, CRP, NO, eNOS and iNOS in rat endotheliocytes.** IL-6, TNF-α, CRP, NO, eNOS and iNOS in rat endotheliocytes prior to and following CIH induction are presented in Table VII and Fig. 6. During the 3 h of CIH induction, IL-6, TNF-α, CRP and iNOS in rat endotheliocytes increased, while NO and eNOS decreased, gradually and significantly (P<0.05).
Discussion

Comparison between Hakka CIH patients and non-Hakka CIH patients. According to the analysis of hemodynamic alterations, the patients with CIH exhibited hypertension. From analysis of the clinical data in the patients with CIH combined with hypertension, it was observed that older people exhibited an increased incidence, as did males. The results of the analysis of BMI and T2DM history indicated that obesity was associated with an increased incidence of CIH combined with hypertension. However, the BMI of the non-Hakka patients was decreased, which indicated that the Hakka patients with increased BMI may be at increased risk of CIH combined with hypertension. Obesity results in excessive fat around the neck, a softened airway and relative macroglossia, resulting in snoring (23). It was hypothesized that certain factors may protect the Hakka people, even with their higher average BMI, from obesity. In addition, individuals who smoke may be at an increased risk of CIH combined with hypertension, demonstrating that smoking is a risk factor for CIH.

Through hemodynamic analysis, it was observed that patients with CIH exhibited increased SAP, DAP, MAP and HR compared with healthy people. However, the non-Hakka patients exhibited increased SAP compared with Hakka patients, suggesting that the Hakka people may have a decreased SAP in CIH combined with hypertension, or that there are other factors influencing their SAP.

Through the comparisons of serum markers, it was demonstrated that patients with CIH combined with hypertension exhibited increased serum IL-6, TNF-α and CRP levels, and decreased NO and NOS levels, compared with healthy volunteers. When comparing between different patients, the IL-6 and TNF-α levels were increased in non-Hakka patients compared with Hakka patients, while the NO level was decreased. The results of the present study indicated decreased inflammation in Hakka patients with CIH combined with hypertension. Therefore, there may be factors which protect the Hakka people from inflammation.

It was previously demonstrated that CIH may stimulate neuromodulatory adaptive changes, influencing the control of breathing, the autonomic nervous system and the cardiovascular system (24). CIH may present with interstitial lung disease (25) and sleep-disordered breathing (26). In addition, CIH has been observed to occur in Hakka patients with hepato-pulmonary syndromes (27). Increasing attention has been paid to the association between CIH and breathing disorders associated with sleep, including obstructive sleep apnea (OSA) (28). OSA is associated with a number of cardiovascular conditions, and one of the closest associations was observed with systemic hypertension (29). The majority of patients with OSA exhibit sleep disruption, which may induce sympathetic disorders of nerve activity and blood pressure (30). The results of the present study indicated decreased inflammation in Hakka patients with CIH in different populations. These data may provide novel insights into the diagnosis, treatment and prevention of CIH, particularly in different populations.

CIH models. In previous studies, the important role of hypoxia in promoting an increase in blood pressure was investigated in humans (31) or by establishing animal models (32). There are a number of advantages to using animal models, including the fact that a single component may be evaluated accurately in a homogeneous population (33). A CIH model in rats was established in the present study. By detecting the SAP, DAP, MAP and HR

Table V. Correlation coefficients of IL-6, TNF-α, CRP, NO and NOS with SAP, DAP, MAP and HR in CIH rats.

| Variable | SAP      | DAP      | MAP      | HR        |
|----------|----------|----------|----------|-----------|
| IL-6     | 0.572b   | 0.439b   | 0.640b   | 0.530b    |
| TNF-α    | 0.673b   | 0.519b   | 0.436a   | 0.448a    |
| CRP      | 0.397a   | 0.418b   | 0.607b   | 0.528b    |
| NO       | -0.568b  | -0.372a  | -0.406b  | -0.630b   |
| NOS      | -0.473a  | -0.460b  | -0.524b  | -0.581b   |

αP<0.05; βP<0.01. CIH, chronic intermittent hypoxia-hypertension; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; NO, nitric oxide; NOS, nitric oxide synthase; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure; HR, heart rate.

Table VI. Apoptosis, IL-6, TNF-α, CRP, NO and iNOS in rat lymphocytes in rats with or without CIH.

| Variable    | CIH       | Control   |
|-------------|-----------|-----------|
| Apoptosis rate, % | 18.59±2.83a | 29.46±1.70 |
| IL-6, ng/ml   | 2.84±0.15a | 0.77±0.11  |
| TNF-α, pg/ml  | 1.73±0.08a | 0.38±0.06  |
| CRP, µg/ml    | 1.95±0.07a | 0.65±0.07  |
| NO, µM        | 4.04±0.21a | 2.38±0.14  |
| iNOS, U/ml    | 0.73±0.05a | 0.10±0.03  |

αP<0.05 vs. control. CIH, chronic intermittent hypoxia; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; NO, nitric oxide; iNOS, inducible nitric oxide synthase.
at different timepoints, it was successfully confirmed that CIH was able to induce hypertension. The SAP, DAP, MAP and HR increased gradually, which indicated that CIH may promote an increase in blood pressure in rats in a time-dependent manner.

The CIH rats were less active and exhibited a decreased appetite, resulting in decreased weight gain compared with healthy rats. OSA was not detected in CIH rats in the present study; it was hypothesized that there were sleep-related disorders in the CIH rats, although this requires further investigation.

In a previous study (34), it was demonstrated that OSA may cause repetitive episodes of airway occlusion with hypoxia, hypercapnia, and alterations in intrathoracic pressure, which may lead to diverse autonomic, humoral, neurohumoral and hemodynamic responses. Indeed, serum IL-6, TNF-α and CRP have been observed to be elevated in patients with OSA (35,36), although there is little evidence to identify a direct association between serum IL-6, TNF-α and CRP, and

Figure 4. Apoptosis of rat lymphocytes in the CIH or control groups. (A) Rat lymphocytes in the CIH group (original magnification, x100). (B) Apoptosis of rat lymphocytes in the CIH group (original magnification, x100; 535 nm). (C) Rat lymphocytes in the control group (original magnification, x100). (D) Apoptosis of rat lymphocytes in the control group (original magnification, x100; 535 nm). (E) Comparison between apoptosis of rat lymphocytes. *P<0.05 vs. control. CIH, chronic intermittent hypoxia.

Figure 5. Alterations in IL-6, TNF-α, CRP, NO and iNOS in rat lymphocytes in the CIH or control groups, assessed by western blotting analysis. *P<0.05 vs. control. CIH, chronic intermittent hypoxia; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; NO, nitric oxide; iNOS, inducible nitric oxide synthase.
Table VII. IL-6, TNF-α, CRP, NO, eNOS and iNOS in rat endotheliocytes prior to and following CIH induction.

| Factor          | Groups | 0 h   | 1 h    | 2 h    | 3 h    |
|-----------------|--------|-------|--------|--------|--------|
| IL-6, ng/ml     | CIH    | 0.72±0.08 | 1.24±0.09<sup>a</sup> | 1.85±0.05<sup>a,b</sup> | 2.34±0.05<sup>a,c</sup> |
|                 | Control | 0.65±0.04 | 0.79±0.08 | 0.84±0.04 | 0.95±0.02 |
| TNF-α, pg/ml    | CIH    | 0.83±0.07 | 1.16±0.06<sup>b</sup> | 2.05±0.08<sup>c</sup> | 2.18±0.06<sup>c</sup> |
|                 | Control | 0.62±0.03 | 0.68±0.09 | 0.54±0.05 | 0.86±0.06 |
| CRP, µg/ml      | CIH    | 0.79±0.03 | 0.95±0.08<sup>b</sup> | 1.04±0.07<sup>b</sup> | 1.27±0.05<sup>d</sup> |
|                 | Control | 0.82±0.05 | 0.80±0.08 | 0.72±0.05 | 0.66±0.03 |
| NO, µM          | CIH    | 3.54±0.26 | 2.88±0.14<sup>a,b</sup> | 1.60±0.22<sup>a,c</sup> | 1.04±0.19<sup>d</sup> |
|                 | Control | 3.92±0.33 | 4.25±0.27 | 3.51±0.22 | 4.07±0.30 |
| eNOS, U/ml      | CIH    | 2.38±0.09 | 0.82±0.04<sup>b</sup> | 0.46±0.05<sup>c</sup> | 0.30±0.03<sup>d</sup> |
|                 | Control | 2.06±0.05 | 1.73±0.08 | 1.80±0.06 | 1.62±0.04 |
| iNOS, U/ml      | CIH    | 0.18±0.05 | 0.26±0.07<sup>a</sup> | 0.38±0.04<sup>b</sup> | 0.40±0.05<sup>b</sup> |
|                 | Control | 0.09±0.01 | 0.07±0.03 | 0.11±0.02 | 0.08±0.02 |

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05 vs. CIH-0 h; <sup>c</sup>P<0.05 vs. CIH-1 h; <sup>d</sup>P<0.05 vs. CIH-2 h. CIH, chronic intermittent hypoxia; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase.

Figure 6. Alterations in IL-6, TNF-α, CRP, NO, eNOS and iNOS in rat endotheliocytes prior to and following CIH induction. (A) Rat endotheliocytes (original magnification, x100). (B) Protein levels of IL-6, TNF-α, CRP, eNOS and iNOS in rat endotheliocytes prior to and following CIH induction, as assessed by western blotting. (C) Rat endotheliocytes IL-6. (D) Rat endotheliocytes TNF-α. (E) Rat endotheliocytes CRP. (F) Rat endotheliocytes eNOS. (G) Rat endotheliocytes iNOS.
the effect of CIH on SAP, DAP, MAP and HR. In the present study, it was observed that serum IL-6, TNF-α and CRP levels increased in CIH rats and lymphocytes, in addition to in rat endotheliocytes following CIH induction, compared with the control. Additionally, serum IL-6, TNF-α and CRP were demonstrated to be positively correlated with the incidence of CIH combined with hypertension in humans, in addition to the SAP, DAP, MAP and HR in CIH rats, suggesting that serum IL-6, TNF-α and CRP may be biomarkers of CIH combined with hypertension.

NO and NOS have been demonstrated to be involved in important regulatory on autonomic functions, particularly in regulating the expression of genes associated with hypoxia (37,38). The present study detected the serum NO and NOS in rats at different timepoints, and it was observed that NO and NOS decreased gradually. The present study additionally analyzed the association between NO and NOS, and CIH combined with hypertension, and confirmed that NO and NOS were negatively correlated with SAP, DAP, MAP and HR in CIH rats. The results of the present study indicated that NO and NOS may be taken to be biomarkers for the diagnosis of CIH combined with hypertension.

However, the levels of NO and iNOS were increased in CIH rat lymphocytes compared with control cells, as did iNOS in rat endotheliocytes following CIH induction, while NO and eNOS decreased significantly in rat endotheliocytes following CIH induction. These results illustrated that the serum NO and NOS were predominantly derived from endotheliocytes. Although CIH induction induced the expression of iNOS, the influence on the total NO was less compared with decreasing eNOS expression.

However, there were limitations to the present study. The sample size in the investigation was small, and was focused on the Hakka people of Huizhou City without investigation other regions, which is likely to result in regional differences and inaccuracy of the correlations between CIH combined with hypertension and clinical data, hemodynamic alterations and serum factors. In addition, due to the limited time of the experiments, the rats were only fed for 21 days for the present study. Further investigations with longer experimental time and more measures are required.

In conclusion, age, male gender, BMI, smoking and T2DM history, and serum IL-6, TNF-α and CRP were positively correlated with CIH combined with hypertension, while NO and NOS were negatively correlated with CIH. Hakka patients exhibited less inflammation and OS in CIH combined with hypertension. Serum IL-6, TNF-α, CRP, NO and NOS may be biomarkers for the diagnosis of CIH combined with hypertension, in addition to targets for the treatment or prevention of CIH or hypertension.

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