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Original Article

Adipose tissue hypoxia caused by cyanotic congenital heart disease and its impact on adipokine dysregulation

Xianguo Wang[1], Xinzhong Chen[1], Dongsheng Xia[1], Yan Cao[2], Nianguo Dong[1]

[1]*Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China;* [2]*Tongji College of Pharmacy, Huazhong University of Science and Technology, Wuhan, Hubei, China*

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Abstract: Objective: To investigate the effects of hypoxia induced by CCHD on the expression and secretion of adipokines. Methods: Subcutaneous adipose tissue and serum were obtained from elective cardiac surgery patients. In CCHD group, n = 17; and in ACHD (Acyanosis congenital heart disease) group, n = 15. Vascular endothelial growth factor (VEGF), inflammation-related adipokines, adiponectin (APN) and Endoplasmic reticulum stress (ER stress) markers were measured in mRNA levels. Hypoxia inducible factor 1α (HIF-1α) and ER stress markers were also measured in protein levels. Results: Immunohistochemical staining showed that protein expression level of VEGF was markedly increased relative to acyanotic groups. Adipose tissue was hypoxic in cyanotic groups. Adipose tissue hypoxia (ATH) led to a substantial increase in inflammation-related adipokines, and a remarkable reduction in adiponectin mRNA levels. Endoplasmic reticulum stress markers were activated by hypoxia. ER stress has been linked to inflammation. These changes were partially reversed by the intervention of normoxia. We exposed normal adipose tissue to $CoCl_2$, a hypoxia mimic, and achieved results similar to the CCHD group. Conclusions: Adipose tissue in CCHD is hypoxic. The change of adiponectin and inflammation-related adipokines are partially caused by ER stress.

Keywords: Adipose tissue, hypoxia, adipokines, cyanosis congenital heart disease, endoplasmic reticulum stress

Introduction

White adipose tissue has traditionally been considered a vehicle for the storage of triacylglycerols. Adipose tissue is now recognized as an important endocrine organ, secreting a variety of the adipokines that are involved in energy metabolism, inflammatory response, and cardiovascular functions, such as including TNF-α, IL-1β, IL-6, IL-10, MCP-1 (monocyte chemoattractant protein-1), MIF (monocyte migration inhibitory factor), leptin, resistin, visfatin, and adiponectin [1, 2]. TNF-α, leptin, resistin, and visfatin are associated with insulin resistance and have pro-inflammatory and atherogenic effects [3]. In contrast, adiponectin has anti-diabetic, anti-inflammatory, and anti-atherogenic effects [4]. Lower concentrations of adiponectin are found in patients with diabetes and coronary atherosclerosis [5, 6].

Hypoxia induces a series of adaptive responses, and transcription factor HIF-1α is a key signal in the cellular response to hypoxia. HIF-1α is composed of two subunits, HIF-1α, which is highly induced by hypoxia to yield the functional transcription factor, and HIF-1β, which is constitutively expressed [7]. The HIF-1α gene has been identified in the adipocytes and the cells of the stromal vascular fraction. HIF-1α is predominantly expressed in the stromal vascular fraction of obese subjects [8].

During the development of obesity, adipocytes become hypertrophic and increases in size up to 140-180 μm in diameter. Because the diffusion limit of oxygen is at most 100 μm, hypertrophic adipocytes may have a less than adequate oxygen supply [9]. Many studies have focused on adipose tissue hypoxia in human obesity. These studies have shown hypoxia to be associated with increased expression of inflammatory genes and decreased expression of adiponectin. They have contributed to the pathogenesis of insulin resistance, which links obesity to many complications, such as type 2

diabetes and cardiovascular diseases. Long-term hypoxia caused by the effects of cyanotic congenital heart disease (CCHD) on adipose tissue had only rarely been reported, so we investigated whether adipose tissue in CCHD patients involved changes in adipokine expression. Results showed that hypoxia resulted in increases in TNF-α, IL-6, MCP-1, MIF, vascular endothelial growth factor (VEGF), GRP78 (Glucose-regulated protein 78), CHOP, and ATF-6 (Activating transcription factor 6) mRNA levels. Protein expression of endoplasmic reticulum stress marker GRP78 and hypoxia marker HIF-1α were also increased, but adiponectin expression decreased at the mRNA levels. These effects were reversed upon return to normoxia. This demonstrates that adipose tissue hypoxia in CCHD may have induced stress upon the endoplasmic reticulum stress and then caused decreases in adiponectin levels and extensive changes in the adipokines.

Materials and methods

Patients

Between January 1 and October 30, 2011, 32 children with congenital heart disease, aged 1 month through 4 years, were included in the study. The patients were divided into two groups. The cyanotic group consisted of 17 patients with cyanotic congenital heart disease (10 boys, 7 girls). These patients all had arterial oxygen saturations of below 85%. The acyanotic group was composed of 15 patients with ACHD (9 boys, 6 girls). These patients all had arterial oxygen saturations over 93%. There was no difference in age, sex and medication between two groups. None of them had other diseases at the time of the study. Cardiac diagnoses were made on the basis of clinical and laboratory examinations, including echocardiography and magnetic resonance imaging. Cardiac diagnoses of cyanotic patients were tetralogy of Fallot (n = 9), transposition of great arteries (n = 4), double outlet right ventricle (n = 2) and pulmonary atresia (n = 2). Cardiac diagnoses of acyanotic patients were ventricular septal defect (n = 12), atrial septal defect (n = 2) and patent ductus arteriosus (n = 1). A total of 32 children who met the inclusion criteria constituted the study population. The study was approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Informed consent was obtained from the parents of all subjects before the study began.

Blood samples and tissue collection

Blood samples were drawn from the jugular vein prior to surgery and centrifuged at 300 g at 4°C for 15 minutes. Serum was stored frozen at -80°C. Subcutaneous adipose tissue samples weighing approximately 500-1000 mg were obtained at the manubrium sternum level. They were divided into two portions. One portion was frozen in liquid nitrogen immediately and then kept in a -80°C freezer until preparation of whole cell lysate or extraction of RNA. The other was kept in formaldehyde or HEPES-salts, used for VEGF immunohistochemistry staining (Zhongshan Goldenbridge, China) or tissue culture.

Adipose tissue explant culture

All procedures for AT explant culture were carried out using sterile technique. AT was placed into sterile filtered HEPES-salts buffer immediately transported under aseptic conditions to the laboratory. AT was washed free of lipid and blood clots and then processed further by cutting into 1×1×1 mm^3 pieces, washed. AT explants were cultured (50 mg AT/ml) under an atmosphere of 5% CO_2 in air at 37°C in defined medium (DM) (1:1 DME low/Ham's F-10 containing 5 mM glucose supplemented with 25 mM HEPES, 15 mM $NaHCO_3$, 2 mM glutamine and 100 U/ml penicillin, 0.1 mg/ml streptomycin, plus 1 nM insulin, 30 nM dexamethasone, 3.3 μM D-biotin, 1.7 μM D-pantothenic acid) for culture. Nine adipose tissues from cyanotic patients were cultured for 24 h under normoxia (21% O_2). The adipose tissue of acyanotic patients was treated with $CoCl_2$ for up to 24 h. For $CoCl_2$ treatment, wells incubated without $CoCl_2$ were used as controls; 100 μM $CoCl_2$ was employed in a time course study. Cell viability was assessed by measuring the media glucose, pH, fat cell size, and histology, there were no significantly change over the culture period. All incubations at each time point were performed in replicates of up to three times.

Measurement of adiponectin and adipokines by ELISA

The serum and tissue culture media concentration of Adiponectin, IL-6, TNF-α were measured with the use of an enzyme-linked immunosorbent assay (ELISA) assay (Neobioscience, China). The intra- and inter-assay variance

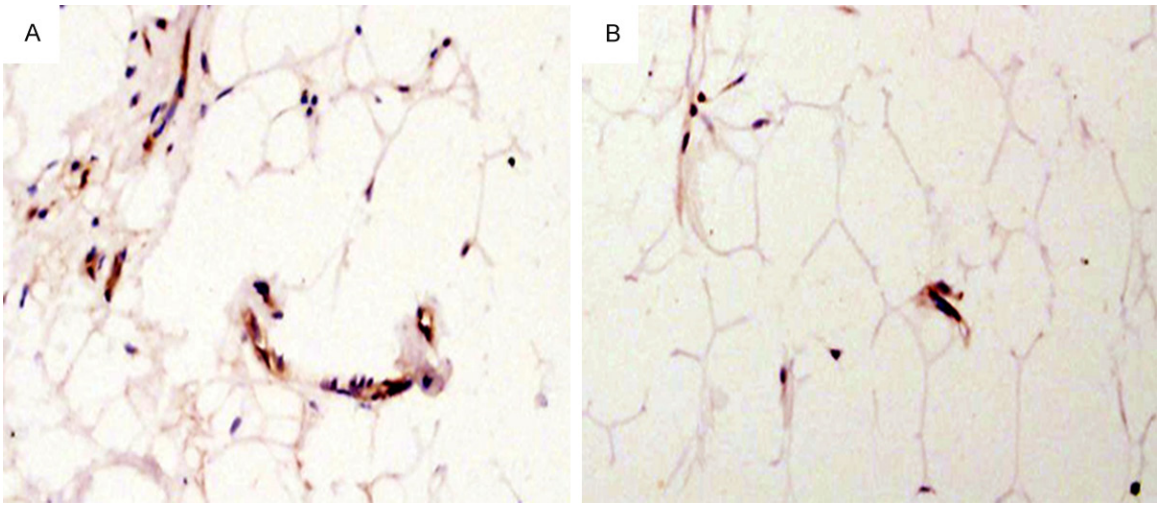

Figure 1. A. Immunocytochemical staining for VEGF in acyanotic group. B. Immunocytochemical staining of VEGF in the cyanotic group. Original magnification, ×200.

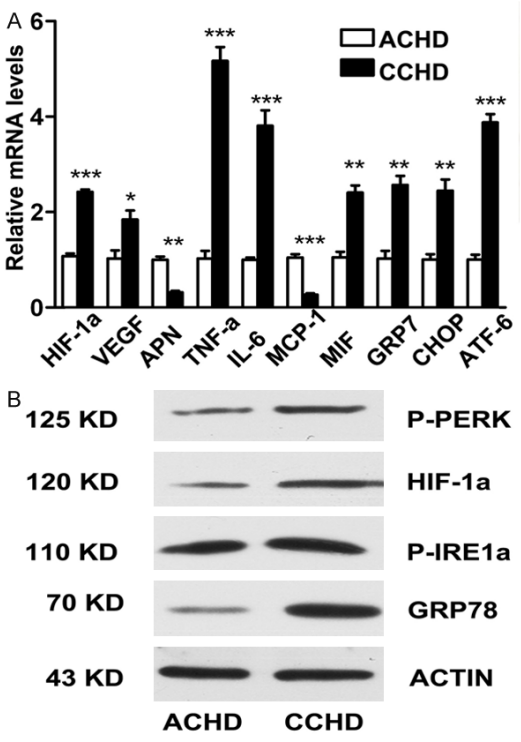

Figure 2. Effects of hypoxia on (A) the mRNA levels of HIF-1α and adipokines and on (B) P-PERK, HIF-1α, P-IRE1 and GRP78 protein secretion in human adipose tissue. Results are mean ± SE (n = 3). In A, **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the acyanotic group. In B, P-PERK, HIF-1α, P-IRE1 and GRP78 protein expression of the cyanotic group relative to the acyanotic group.

coefficients were lower than 9%. The assays were conducted in 96-well microplates according to the manufacturer's instructions. Samples were analyzed in triplicate following the instructions in the kit.

Real-time quantitative RT-PCR

Total RNA was extracted from homogenized fat pads using the TRIzol reagent (Takara, China) according to the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) was conducted using the ABI 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). The following primers were ordered from Invitrogen: HIF-1α forward GACTCAAAGCGACAGATAACACG; HIF-1α reverse ATTCCAGCAGACTCAAATACAAGA; VEGF forward TTGTTGTGCTGTAGGAAGCTCA; VEGF reverse CAGCTACTGCCATCCAATCG; APN forward AGGCCGTGATGGCAGAGAT; APN reverse TCCAATCCCACACTGAATGCT; TNF-α α forward GGTTCGAGAAGATGATCTGACTG; TNF-α reverse CTGCTGCACTTTGGAGTGATC; IL-6 forward TTGGGTCAGGGGTGGTTATT; IL-6 reverse GTGAAAGCAGCAAAGAGGCA; MCP-1 forward CAAGTCTTCGGAGTTTGGGTTT; MCP-1 reverse CTCATAGCAGCCACCTTCATTC; MIF forward CCGTTTATTTCTCCCCACCAG; MIF reverse TCCACCTTCGCCTAAGAGCC; CHOP forward CCACTCTTGACCCTGCTTCTC; CHOP reverse TGGTTCTCCCTTGGTCTTCC; ATF-6 forward AGTCAGTCCATTTTCAGTCTTGTTC; ATF-6 reverse AAGTTATTCAGTCTCGTCTCCTCG. Human β-actin was used as an endogenous reference. This housekeeping gene demonstrated no significant changes in expression when exposed to $CoCl_2$ and 1% O_2. Typically, amplifi-

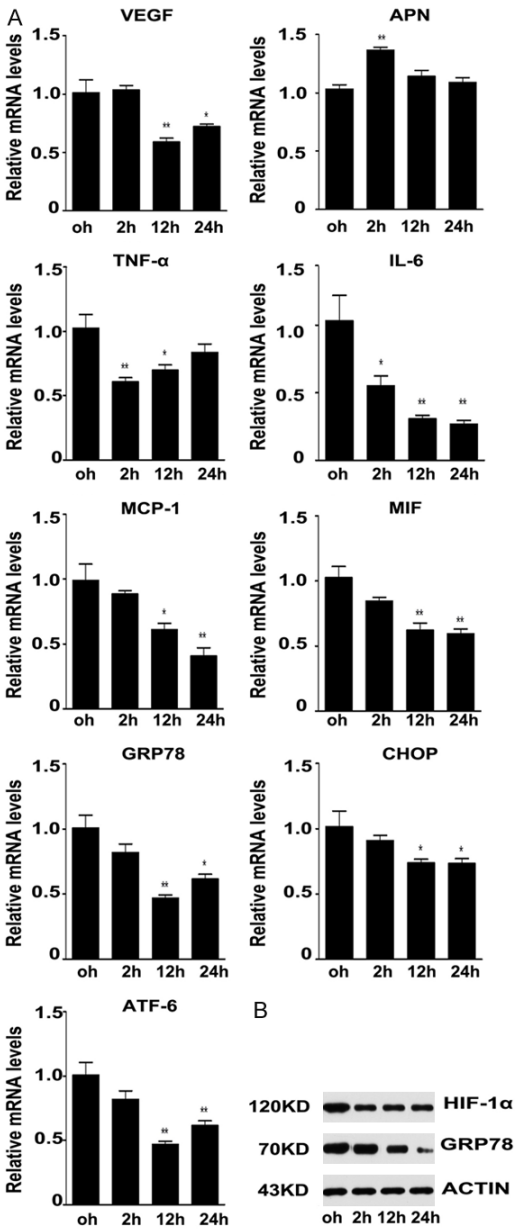

Figure 3. Adipose tissue from the cyanotic group was cultured under normoxic conditions for up to 24 h. A. Relative mRNA levels of VEGF, IL-6, TNF-α, MCP-1, MIF, and ERS markers mRNA levels. B. HIF-1α and GRP78 protein secretion in human adipose tissue. Results are mean ± SE (n = 3). In A, **P*<0.05, ***P*<0.01, compared with 0 h. In B, HIF-1α nd GRP78 protein expression of the cyanotic group relative to 0 h.

cation began with 2 min at 50°C, 2 min at 95°C, and then 40 cycles of the following: 15 s at 95°C, 15 s at 58°C, and 45 s at 72°C. Data were obtained as cycle threshold (Ct) values and used to determine ΔCt values (ΔCt = Ct of gene of interest-Ct of β-actin). The mean value of triplicates was used for relative mRNA level. To exclude bias due to averaging, data were transformed through the Power equation $2^{-\Delta\Delta Ct}$. PCR efficiency was close to 100% in all runs, and all samples were analyzed in at least triplicate.

Western blot analysis

Whole adipose tissue protein was made using homogenization in a lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Triglycerides were removed from the lysate before protein assay. Extraction of nuclear protein and Western blot analysis were conducted as described elsewhere [10]. Western blot analysis was carried out using P-PERK, HIF-1α, P-IRE1 and GRP78 antibodies (Epitomics, U.S.).

Statistical analysis

All of the experiments in this study were conducted at least three times, and results remained consistent. Data from representative experiments are presented. Statistical analyses were performed using SPSS 18.0 software (Statistical Program for the Social Sciences). The correlation values are means SE of multiple data points or samples to represent the final result. Student's t-test and two-way ANOVA were used for statistical analysis of the data. *P* values <0.05 were considered significant.

Results

Serum adipokines in acyanotic and cyanotic groups

Serum adiponectin levels were significantly higher in acyanotic than in cyanotic groups (*P*<0.01) (**Figure 1**). Serum TNF-α values were higher in the acyanotic groups than in the cyanotic groups (*P*<0.01). There was no significant difference in serum levels of IL-6 between the acyanotic and cyanotic groups.

Immunohistochemistry

Immunohistochemical staining of adipose tissue in cyanotic and acyanotic groups was shown in **Figure 1**. VEGF was used as a marker of hypoxia in adipose tissue. It showed that VEGF expression was significantly higher in the cyanotic group.

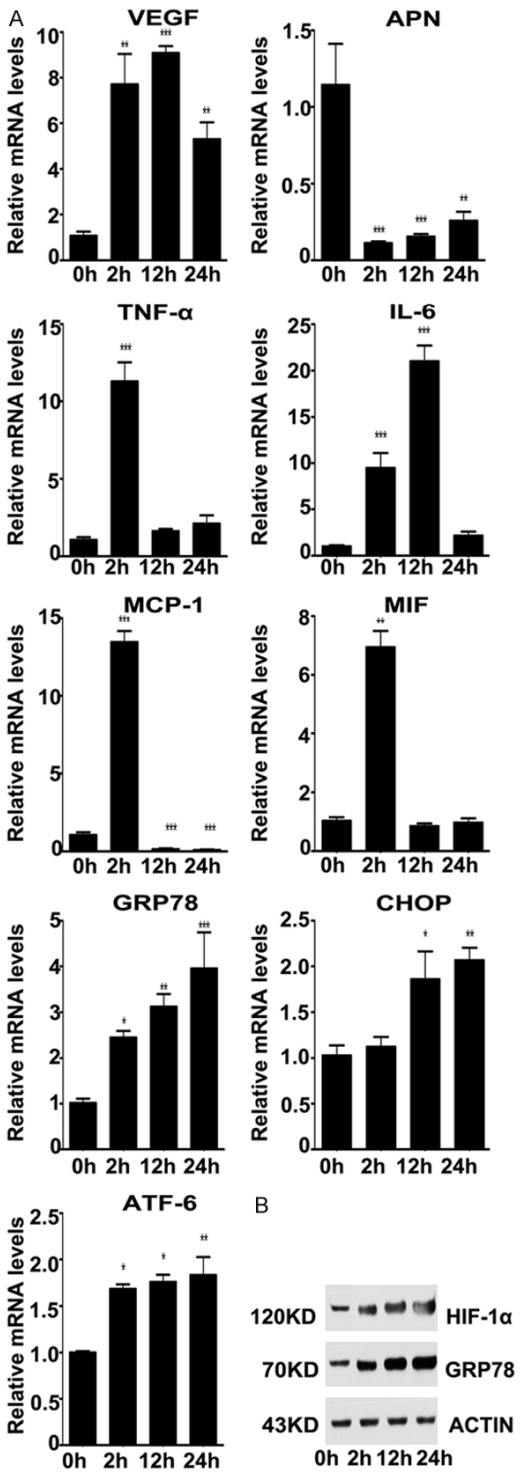

Figure 4. Effects of $CoCl_2$ on (A) the mRNA levels of VEGF and adipokines and on (B) HIF-1α and GRP78 protein secretion in human adipose tissue. Adipose tissue from the acyanotic group was incubated in medium containing 100 μM $CoCl_2$ for 24 h. In A, **P*<0.05, ***P*<0.01, ****P*<0.001 compared with the acyanotic group. In B, HIF-1α and GRP78 prote in expression of the acyanotic group relative to 0 h.

Expression of adipokines in the adipose tissues of the cyanotic and acyanotic groups

In the cyanotic group, the SPO_2 levels of patients were lower than 85% before the operation. The expression of a series of inflammation-related adipokine genes was then examined in the hypoxic adipose tissue. There was a significant reduction in mRNA levels of adiponectin, and MCP-1. Adiponectin levels decreased three to fourfold. In contrast, hypoxia resulted in a marked increase in mRNA levels for TNF-α and IL-6. The increase was about twofold for VEGF and two to fourfold for MIF, GRP78, CHOP, and ATF-6. HIF-1α mRNA levels increased two to threefold. In the cyanotic group, P-PERK, HIF-1α, P-IRE1 and GRP78 protein levels were higher than in the acyanotic group (**Figure 2**).

Adipose tissue in cyanotic group cultured under normoxic conditions

When the adipose tissue in the cyanotic group was restored to normoxic conditions (21% O_2) led to a reversal in VEGF, L-6, TNF-α, MCP-1, and MIF mRNA levels. Levels of GRP78, CHOP, and ATF-6 the mRNA were lower than at 12 h after exposure to normoxic conditions. These changes in adiponectin levels were reversed after 2 h of reexposure to 21% O_2. HIF-1α protein level was lower after 2 h of reexposure to normoxic conditions. However, GRP78 protein level was lower after 12 h of reexposure (**Figure 3**).

Adipose tissue in acyanotic group response to $CoCl_2$ treatment

Hypoxic effects of adipose tissue can be mimicked by the divalent transition metal ion cobalt. Adipose tissue (in the acyanotic group) was treated with $CoCl_2$ at concentrations of 100 μM for 24 h. The expression of inflammation-related adipokines and ERS markers were then examined in the $CoCl_2$-treated adipose tissue. There was a significant reduction in adiponectin mRNA levels. Adiponectin mRNA levels decreased three to eightfold. There was a marked increase in mRNA levels for VEGF, IL-6, TNF-α, MCP-1, and MIF. The increase was four to eightfold for VEGF, and six to twentyfold for IL-6, TNF-α, MCP-1, and MIF. ERS markers GRP78, CHOP, and ATF-6 increased one to twofold (**Figure 4**).

Table 1. Adiponectin, TNF-α, and Il-6 levels of patients with CCHD and ACHD

	Acyanotic patients (n = 15)	Cyanotic patients (n = 17)
Adiponectin (ng/ml)	17.02±3.7	14.72±5.8**
TNF-α (pg/ml)	18.70 ±2.1	20.84±4.5**
Il-6 (pg/ml)	16.22±1.44	16.40±1.27

**P<0.01.

Adipose tissue in acyanotic group response to low O_2 tension

When adipose tissue (in the acyanotic group) was cultured in 1% O_2 up to 24 h, the expression of inflammation-related adipokines and ERS markers were then examined. The results were similar to the above $CoCl_2$ treatment. So we didn't show the results.

Discussion

In obesity, adipocyte diameter increases to up to 140-180 μm as a result of hypertrophy [11]. Because the diffusion limit of oxygen is around 100 μm [12], this hypertrophy leads to the local hypoxic areas in adipose tissue [12, 13]. Adipose tissue hypoxia induces an increase in the synthesis of adipokines, such as IL-1β, IL-6, TNF-α, MCP-1, and MIF which are linked to inflammation, and a reduction of adiponectin production and circulating levels. Recent studies have suggested that adipose tissue hypoxia (ATH) is a root of chronic inflammation in obesity [14]. Hypoxia in CCHD manifests as the reduction of oxygen content in the blood. In this study, we investigated the effects of hypoxia on adipose tissue in CCHD patients.

Our results show that cyanotic patients were shorter and weighed less than acyanotic volunteers. This is consistent with the observations of David et al. [15]. They found no difference in adipocyte size, though total number of adipocytes in the cyanotic children decreased and body fat was reduced due to adipocyte hypocellularity.

Serum TNF-α levels was higher in cyanotic groups than in the acyanotic group and adiponectin was lower in cyanotic groups (P<0.01) (**Table 1**). There was no significant difference in IL-6 levels. However, Yilmaz et al. have shown that TNF-α levels are significantly higher in both acyanotic patients and cyanotic patients than in healthy people, but there was no significant difference between acyanotic patients and cyanotic patients with respect to TNF-α levels [16]. This discrepancy might be due to different choices made by the patients and needs to be further clarified.

The transcription factor HIF-1α is a key signal in the cellular response to hypoxia, the α-subunit of which is highly induced by hypoxia. HIF-1α protein is stabilized by hypoxia and degraded by an ubiquitin-dependent proteasome under normoxic conditions. HIF-1α gene expression was found to be increased throughout the adipose tissues of obese subjects, predominantly in the stromal vascular fraction. However, its expression decreased after weight loss [17]. Poulain-Godefroy et al. suggested that it is important to designate an amount of HIF-1α protein, rather than an amount of mRNA, as a key marker in studies examining whether adipose tissue hypoxia occurs in human obesity. In cyanotic groups, VEGF expression (**Figure 1**) and hypoxia marker HIF-1α protein (**Figure 2**) were markedly higher than in acyanotic groups. According to these studies, we speculated that adipose tissue was hypoxic in cyanotic groups. Our study also showed that adipose tissue showed increased expression of a number of adipokines linked to inflammation, including TNF-α, IL-6, MCP-1, MIF, and decreased levels of adiponectin mRNA expression (**Figure 2**). Levels of mRNA coding for endoplasmic reticulum stress markers GRP78, CHOP, and ATF-6 were increased. These effects were partially reversed upon return to normoxia (**Figure 4**).

$CoCl_2$, a known inducer of HIF-1α, has been widely employed as a hypoxia mimic, which is thought to act mainly by inhibiting the oxygen- and Fe^2+-dependent enzyme, HIF-1α Aprolyl-4-hydroxylase, leading to HIF-1α stabilization and the activation of cellular hypoxia-dependent pathways under normal oxygen levels. $CoCl_2$ has been used to examine the effects of chemically induced hypoxia on adipose tissues in ACHD. Our results show significantly increases in endoplasmic reticulum stress markers, accumulation of HIF-1α, increased expression of inflammation-related adipokine expression in mRNA levels and decreased adiponectin (**Figure 3**). Other studies have also show that $CoCl_2$ induces HIF-1α expression in various cell

types, including cancer cells and brown adipocytes [18].

Various stimuli can trigger ER stress. These stimuli include glucose and nutrient deprivation, hypoxia, and increased synthesis of secretory proteins [19]. To cope with ER stress, cells trigger a set of pathways known as unfolded protein response (UPR). This response is mediated by RNA-dependent protein-kinase-like ER eukaryotic translation initiation factor 2-kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor 6 (ATF6) [20]. UPR causes increased transcription of genes involved in ER protein folding and slow protein synthesis at the translation level, alleviating the ER load [21]. ER stress and the UPR are linked to inflammation via several distinct mechanisms. IRE-1a can activate JNK and NFκB pathways. The NFκB pathway may also be activated through PERK signaling. JNK upregulates the expression of inflammatory genes through activation of the AP-1 transcription factor complexes [22]. IKK-NFκB pathway can induce the expression of multiple inflammatory genes, such as TNF-α and IL-6 [23]. CHOP is downstream of PERK. GRP78 was used to indicate the degree of ERS. ATF-6 in mRNA level and IRE-1a in protein level were measured in this study. Results show that the expression of ATF-6 and IRE-1a increased in hypoxic adipose tissue. Induction of UPR in endothelial cells has been reported to cause increased expression of inflammatory genes, including TNF-α, IL-6, MCP-1, and IL-8 [24]. It has been reported that exposure of 3T3-L1 adipocytes to hypoxia dysregulated the expression of adipocytokines such as adiponectin and plasminogen activator inhibitor type-1 and increased expression of the mRNAs of ERS marker genes, CHOP and GRP78. This effect was mediated by ERS and posttranscriptional regulation [25].

Unlike adiponectin, hypoxia stimulated the expression of the genes encoding TNF-α, IL-6, MCP-1, MIF, and VEGF. VEGF is activated by hypoxia. An increase in VEGF mRNA levels was observed in adipose tissues in CCHD. This was consistent with studies on rat white adipose tissue [26]. Hypoxia and treatment with $CoCl_2$ could cause transitory increases in MCP-1 mRNA, which is an important factor in macrophage recruitment. MIF, a pro-inflammatory agent, was also stimulated by hypoxia. The expression of classic inflammatory factors TNF-α and IL-6 was upregulated by hypoxia.

The adipose tissues were used in the present study, because, firstly, in white adipose tissues, the preparation and incubation of different cell fractions may alter their relative rates of adipokine production, and adipocytes engaged in crosstalk with non-adipocyte fractions. Second, we believe that adipose tissues obtained from patients reflected the real situation in disease conditions. Third, some studies were performed on 3T3l-1 adipocytes and mice exposed to hypoxia [25]. It was difficult to make quantitative comparisons regarding the extent to which various cell types contributed quantitatively to the overall production of inflammatory factors, though in vitro studies demonstrated that mature adipocytes were less important than the non-adipocyte fraction in the production and release of most cytokines and chemokines [27]. We obtained subcutaneous adipose tissue samples at the manubrium sterni level. Whether adipose tissues from different sites undergo the same change merits further investigation.

In summary, we demonstrated that adipose tissues in CCHD are hypoxic and that this hypoxia dysregulates the production of adipocytokines in those tissues during CCHD. This effect is partially mediated by ER stress. These changes were partially reversed by normoxia intervention. If patients with CCHD undergo surgery in time and normal oxygen partial pressure is restored in the blood, some of the inflammation-related adipokine dysfunction may be reversed; the patient quality of life may be improved. Collectively, our results suggest that adipose tissue hypoxia is responsible for decreases in adiponectin levels and inflammation-related changes in adipokine levels.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xinzhong Chen, Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei, China. Tel: 86-27-85351610; Fax: 86-27-85351636; E-mail: xinzhong_chen@sina.com

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