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間歇性缺氧對心肌擴張及心肌凋亡之探討：加鐵氧化傷害加乘長期間歇性缺氧誘發心肌擴張及凋亡研究(I)；探討短暫和長期間歇性缺氧對心臟細胞凋亡及存活訊息途徑之影響差異(II)

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Short-Term versus Long-Term Intermittent Hypobaric Hypoxia on Cardiac fibrosis and Fas Death Receptor Dependent Apoptotic Pathway in Rat Hearts

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Short Running Head: Fas apoptotic pathway and Hypoxia

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Abstract

Background. Short-term versus long-term intermittent hypoxia had been shown to exert cardiac protective versus deleterious effects. It is unclear if short-term versus long-term intermittent hypobaric hypoxic challenges exert opposite effects on Fas death receptor-dependent apoptotic pathway in rat hearts. ***Methods.*** Seventy-two Sprague-Dawley rats were randomized assigned into two groups: first, short-term intermittent hypobaric hypoxia (STIHH)-normobaric normoxia (n=12), hypobaric hypoxia (380 mmHg, 12% O_2, 8 hrs/day) for 1 day (n=12), and for 4 days (n=12) and second, long-term intermittent hypobaric hypoxia (LTIHH)-normobaric normoxia (n=12), hypobaric hypoxia for 1 week (n=12) and 2 weeks (n=12). After STIHH or LTIHH challenge, Fas receptor-related pathway and histopathological analysis in the excised left ventricle was determined by Western blotting, RT-PCR, Hematoxylin-eosin staining, and Masson trichrome staining. ***Results.*** Pro-apoptotic Fas death receptors and TNFα mRNA were significantly decreased after STIHH and Fas ligand, Fas associated death domain (FADD), activated caspase 8 were not changed after STIHH whereas Fas receptor, TNFα, FADD, and caspase 8 were increased after LTIHH. In addition, cardiomyocyte disarray and fibrosis were observed in 1 week LTIHH as well as cardiac hypertrophy and more severe disarray and fibrosis were observed in 2 week LTIHH. ***Conclusions.*** STIHH appeared to exert protective effects on hearts such as downregulation of TNFα mRNA and Fas receptor whereas LTIHH appeared to exert deleterious and pro-apoptotic effects such as upregulation of TNFα and Fas-mediated apoptotic pathways and lead to cardiac fibrosis, which imply that cardiac protective or pro-apoptotic effect exerted by intermittent hypobaric hypoxia is tightly time-course dependent.

Key words: cardiac fibrosis, cell apoptosis, high altitude, Fas pathway, time course

Introduction:

Apoptosis, a physiological program of cellular death, may contribute to many cardiac disorders, such as post-infarction myocardial apoptosis and heart failure [1-3]. Fas also known as APO-1/CD95 is a tumor necrosis factor (TNF) receptor superfamily member and Fas death receptor-induced apoptotic pathway was thought to be one of the major pathway directly to triggers cardiac apoptosis [1, 4]. This pathway was initiated by death receptor agonists, including Fas ligand (FasL) and TNF-α [4]. Fas ligand binding followed by Fas-receptor oligomerisation led to formation of a death-inducing signal complex starting with recruitment of the the Fas-associated death domain (FADD) of the adaptor protein [4]. Fas receptor oligomerization results in the activation of caspase 8 and causes the activation of apoptosis [5, 6]. After the occurrence of apoptosis or in the end-stage heart failure, the remodeling of the failing myocardium was associated with excessive collagen deposition and fibrosis [3, 7]. Besides, the collagen synthesis appears to replace the space of damaged cardiomyocytes in cardiomyopathy [3, 7]. Hence, the cardiac fibrosis following myocardial apoptosis is recognized as a predictor of adverse outcomes in subjects with cardiomyopathy [3, 8]. Therefore, the evaluation of apoptosis and/or fibrosis should be an important issue of predicting the development of hypoxia-induced cardiac abnormality.

Hypoxia-induced cardiomyocyte apoptosis or post-infarction myocardial apoptosis has been associated with Fas receptor dependent apoptotic pathway [2, 9-11]. In contrast, intermittent hypoxia has been shown to provide myocardial protection against ischemia/reperfusion-induced injury and attenuated ischemia/reperfusion-induced apoptosis [12]. To clarifying the controversial findings, our previous study presented opposite effects on Bcl2 family, which showed that

anti-apoptotic Bcl-2 proteins were increased after 1 day and 4 day short-term intermittent hypobaric hypoxia (STIHH) but decreased after 1 week and 2 week long-term intermittent hypobaric hypoxia (LTIHH). In contrast, pro-apoptotic BNIP3 and Bad proteins were significantly decreased after STIHH but increased after LTIHH [13]. Our previous study showed that STIHH appeared to exert protective effects on hearts whereas LTIHH appeared to exert deleterious effects. However, it is unclear if short-term versus long-term intermittent hypobaric hypoxic challenges exert opposite effects on Fas death receptor dependent apoptotic pathway and cardiac fibrosis in rat hearts.

In the current study, to reveal the controversial effects of intermittent hypobaric hypoxia, Fas ligand, TNF-α, FADD, caspase 8, and interstitial fibrosis in two groups, STIHH and LTIHH, in the excised left ventricle were determined by Western blotting, RT-PCR, and histopathological analysis stained with hematoxylin-eosin and Masson trichrome. We hypothesized that short-term and long-term intermittent hypobaric hypoxic challenges may exert opposite effects on Fas death receptor dependent apoptotic pathway and cardiac fibrosis in rat hearts.

Materials and Methods

Animal model

Male Sprague Dawley rats weighting 350~400 g at 12 weeks old were purchased from National Science Council Animal Center, Taiwan. All rats were housed three per cage. Ambient temperature was maintained at 25ºC and the animals were kept on an artificial 12-h light-dark cycle. The light period began at 7:00 A.M. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and water *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University, Taichung, Taiwan, and the principles of laboratory animal care (NIH publication) were followed.

Short-term versus long-term hypobaric hypoxia exposures

A total of 72 rats were randomly divided into 2 groups, short-term intermittent hypobaric hypoxia (STIHH) versus long-term intermittent hypobaric hypoxia (LTIHH). Both the STIHH and LTIHH groups were then randomly subdivided into 3 groups each as follows: 1) STIHH group- normobaric normoxia (760 mm, 21% O_2 and 79% N_2) for 1 or 4 days (n=12), hypobaric hypoxia (380 mm, 12% O_2 and 88% N_2, for eight hours per day) for 1 day (n=12), and hypobaric hypoxia for 4 days (n=12); 2) LTIHH group-normobaric normoxia for 1 or 2 weeks (n=12), hypobaric hypoxia for 1 week (n=12), and hypobaric hypoxia for 2 weeks (n=12). (Fig 1) After normoxic or hypoxic exposure, rats were weighed and decapitated. The eight hearts of animals per subgroup were excised and cleaned with ddH_2O. The left and right atrium and ventricle were separated and weighed. The ratios of the total heart weight and the left ventricular weight to body weight were weighed and calculated.

Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) at a ratio of 100 mg tissue/0.5 ml PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -70°C for further experiments.

Electrophoresis and Western Blot

The tissue extract samples were prepared as described by homogenizing with buffer. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was done with 10% polyacrylamide gels. The samples were electrophoresed at 140 V for 3.5 hours and equilibrated for 15 min in 25 mM Tris-HCl, pH8.3, containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred to nitrocellulose membranes (Amersham, Hybond-C Extra Supported, 0.45 Micro) using a Bio-rad Scientific Instruments Transphor Unit at 100 mA for 14 h. Nitrocellulose membranes were incubated at room temperature for 2 hours in blocking buffer containing 100 mM Tris-HCl, pH7.5, 0.9% (w/v) NaCl, 0.1% (v/v) fetal bovine serum. Monoclonal antibodies including Fas ligand, Fas, FADD, caspase 8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (Neo Markers, Fremont, CA, USA) were diluted 1:200 in antibody binding buffer containing 100 mM Tris-HCL, pH7.5, 0.9%(w/v) NaCl, 0.1%(v/v) Tween-20 and 1%(v/v) fetal bovine serum. Incubations were performed at room temperature for 3.5 hours. The immunoblots were washed three times in 50 ml blotting buffer for 10 min and then immersed in the second antibody solution containing alkaline phosphatase goat anti-rat IgG (Promega) for 1 hour and diluted 1000-fold in binding buffer. The immunoblots were then washed in blotting buffer for 10min three times. Color development was presented in a 20 ml mixture

consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3-indolyl-phosphate, 100 mM NaCl and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5.

RNA Extraction

Total RNA was extracted using the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) according to the manufacturer's instructions. Each heart was thoroughly homogenized in 1 ml Ultraspec reagent/100 mg tissue using a Polytron homogenizer. The homogenates were washed twice with 70% ethanol by gentle vortexing. RNA precipitates were then collected by centrifugation at 12,000 *g* and dried under vacuum for 5-10 min before dissolving in 50 µl diethylpyrocarbonate-treated water, and then incubated at 55-60°C for 10-15 min.

RT-PCR

Total RNA was reverse transcribed and then amplified by the polymerase chain reaction using a Super Script Preamplification System for first strand cDNA Synthesis and Taq DNA polymerase (Life Technologies [GIBCO BRL], Rockville, Maryland, USA). RT-PCR products (45µl) were separated on a 1.25% agarose gel (Life Technologies [GIBCO BRL]). Amplimers were synthesized by MdBio, Inc. based on cDNA sequences from Gen Bank. The rat GAPDH was used as an internal standard. The following rat primers were used: Rat TNFα forward primer-TCGAG TGACA AGCCC GTAG; Rat TNFα reverse primer- CAGAG CAATG ACTCC AAAGT AGAC; Rat GAPDH forward primer-GGGTG TGAAC CACGA GAAAT; Rat GAPDH reverse primer-CCACA GTCTT CTGAG TGGCA. Densitometric analysis of immunoblots and PCR was performed using AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro,. CA, USA).

Hematoxylin-eosin and Masson trichrome staining

After the hearts were removed from four rats per subgroup, they was soaked in

formalin and covered with wax. Slides were prepared by first soaking for dehydration. They were passed through a series of graded alcohols (100%, 95% and 75%), 15 minutes of each. The slides were then dyed with hematoxylin and eosin or Masson trichrome. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 minutes each. At the end, they were soaked with Xylene I- Xylene II. Photomicrographs were obtained using Zeiss Axiophot microscopes.

Statistical Analysis

The data were compared among groups of animals in either short-term intermittent hypobaric hypoxia (STIHH)-three subgroup or long-term intermittent hypobaric hypoxia (LTIHH)-three subgroup using one-way analysis of variance (ANOVA) with pre-planned contrast comparison. In all cases, a difference at $P<0.05$ was considered statistically significant.

Results

Cardiac changes of rats under short-term versus long-term intermittent hypobaric hypoxia

The heart weights and ventricular weights were not significantly changed following 1-day and 4-day short-term intermittent hypobaric hypoxia (STIHH) (Table 1). The heart weights and ventricular weights were not significantly increased following 1 week long-term intermittent hypobaric hypoxia (LTIHH) whereas after 2 week LTIHH, significant cardiac hypertrophy was observed due to increased heart weight-to-body weight ratio (Table 2).

Changes of Fas death receptor protein levels under short-term versus long-term intermittent hypobaric hypoxia in the cardiac tissues

To further understand the Fas ligand and Fas receptor associated with the Fas receptor dependent apoptotic pathway induced by short-term versus long-term hypobaric hypoxia, the protein levels of the Fas ligand and Fas death receptor were measured by Western Blotting. Fas death receptor protein levels were significantly decreased following 1-day and 4-day STIHH (Fig 2A), but in contrast were significantly increased following 1-week and 2-week LTIHH (Fig 2B). However, Fas ligand protein levels were not changed following 1-day and 4-day STIHH or 1-week and 2-week LTIHH (Fig 2).

Changes of TNF-α mRNA expression under short-term versus long-term intermittent hypobaric hypoxia in the cardiac tissues

To further understand another ligand associated with the Fas receptor dependent apoptotic pathway induced by short-term versus long-term hypobaric hypoxia, the gene expressions of the TNF-α were measured by RT-PRC. TNF-α mRNA expression was significantly decreased following 1-day and 4-day STIHH (Fig 3A), but in

contrast were significantly increased following 1-week and 2-week LTIHH (Fig 3B).

Changes of Fas-associated death domain (FADD) under short-term versus long-term intermittent hypobaric hypoxia in the cardiac tissues

To further understand the common domain, FADD associated with the Fas receptor dependent apoptotic pathway induced by short-term versus long-term hypobaric hypoxia, the FADD protein levels were measured by Western Blotting. FADD protein levels were not changed following 1-day and 4-day STIHH (Fig 4A), but were significantly increased following 1-week and 2-week LTIHH (Fig 4B).

Changes of activated caspase 8 under short-term versus long-term intermittent hypobaric hypoxia in the cardiac tissues

To further understand the Fas-mediated caspase activation induced by short-term versus long-term hypobaric hypoxia, the activated caspase-8 protein levels were measured by Western Blotting. Caspase-8 protein levels were not changed following 1-day and 4-day STIHH (Fig 5A), but were significantly increased following 1-week and 2-week LTIHH (Fig 5B).

Cardiomyopathic alteration of rat hearts under long-term intermittent hypoxia

To understand the possible cardiomyopathic alteration after intermittent hypoxia, histopathological analysis of ventricular tissues with hematoxylin and eosin staining and Masson trichrome staining was performed. The ventricular myocardium under normbaric normoxia showed normal architecture with minimal interstitial fibrosis (Fig 6A, Fig 6B). However, abnormal myocardial architecture and increased interstitial space were observed in rat hearts after 1-week LTIHH, and those observations become more obvious after 2-week LTIHH in 100 X magnification images (Fig 6A). Besides, hearts stained with Masson trichrome showed extensive fibrosis, increased collagen deposition, and myofibril disarray in rats after 1-week and

showed more obvious after 2-week LTIHH in x 200 magnification images (Fig 6B). No obvious differences of myocardial architecture, interstitial spaces, and fibrosis were observed following 1-day and 4-day STIHH (imagines not shown).

DISCUSSION

Major findings

Our main findings can be summarized as follows: Decreased Fas death receptor, decreased TNF-α gene expression, unchanged Fas ligand, FADD, and activated-caspase 8 were found in rat hearts following 1-day and 4-day STIHH, but in contrast Fas ligand, Fas death receptor, TNF-α gene expression, FADD, activated-caspase 8, and even cardiac interstitial fibrosis were all significantly increased following 1-week and 2-week LTIHH. Our major findings imply that short-term versus long-term intermittent hypobaric hypoxia exerted opposing effects on cardiac Fas receptor dependent apoptotic pathways, protective versus deleterious (pro-apoptotic or fibrotic) effects, on rat hearts.

Opposing effects on cardiac Fas death receptor dependent apoptotic pathway

The type I or 'extrinsic' apoptotic pathway is mediated by external factors that bind to members of the death receptor superfamily, such as Fas. In type I apoptosis, Fas death receptors respond to specific ligand binding by Fas ligand or TNF-α by recruiting specific intercellular adaptor proteins (e.g. FADD) through homologous 'death domains'. FADD couples directly aggregates procaspase 8, leading to the cleavage and activation of caspase 8, which in turn induces cell apoptosis [4]. In the current study, decreased Fas death receptor and TNF-α gene expression but unchanged Fas ligand, FADD, activated-caspase 8, and interstitial fibrosis after 1-day and 4-day STIHH imply that STIHH might exert protective effects on Fas death receptor dependent apoptotic pathway and appeared to prevent from activation of caspase 8 and cardiac fibrosis. The results in the STIHH group further support our previous findings short-term intermittent hypobaric hypoxia exerted protective effects

on rat hearts, which showed that pro-apoptotic Bcl-2 family members, BNIP3 and Bad were significantly decreased whereas anti-apoptotic Bcl-2 family, Bcl2 were significantly increased after STIHH [13]. Our current study is the first time to demonstate downregulation of Fas death receptor and TNF-α gene expression after STIHH but upregulation of Fas death receptor and TNF-α gene expression after LTIHH.

In contrast, overall increases in Fas ligand, Fas death receptor, TNF-α gene expressions, FADD, activated-caspase 8, and cardiac instertitial fibrosis after 1-week and 2-week LTIHH imply that LTIHH activated Fas death receptor dependent apoptotic pathway and led to cardiac fibrosis. The results in the LTIHH group are consistent with the notion that Fas-Fas ligand signaling pathway is activated in response to the different severity or various timing of hypoxia or ischemia/reperfusion [11, 14-16]. The following three studies may indirectly support our finding after LTIHH. The mRNA levels of Fas ligand, Fas death receptor, and activated caspases 8 were upregulated by simulated ischemia/reperfusion, which was created by withdrawing serum from the culture medium and placing the cardiomyocytes in a hypoxic chamber (<1% O_2, 5% CO_2, 37 °C) for 12 h, and then returning the cells to normal culture conditions (21% O_2, 5% CO_2, 37 °C) for an additional 4 or 12 h [14]. In an isolated Langendorff perfused rat heart model, ischemia and reperfusion induces the activation of caspase-8 in the heart [16]. In cardiomyocytes with or without genetic manipulations, FADD/caspase-8 signaling was suggested as a primary role for apoptosis of cardiomyocytes subjected to ischemia or hypoxia/serum deprivation [17].

There is an association between the turnover of collagens and remodeling of the rat ventricles [3, 7]. The remodeling progresses immediately after myocardial damage with an increased level of collagenases [18]. The collagens synthesized by the

fibroblasts will invade and replace the apoptotic myocytes [3, 7, 8, 19]. The myocardial interstitial changes resulted from increased collagen deposition lead to cardiac stiffness and cardiac dysfunction [19]. Accordingly, the accumulated collagens will further contribute to the development of ventricular fibrosis and heart failure [8]. In our findings, abnormal myocardial architecture, increased interstitial space, increased cardiac fibrosis following 1-week and 2-week LTIHH suggest the development of cardiomyocyte death characterized by the distortion in myocardium architecture and cardiac fibrosis.

Significance and clinical application

Our current findings combing with previous findings in Bcl2 apoptotic pathway [13] strongly suggest that intermittent hypobaric hypoxia exerted opposing apoptotic and/or fibrotic effects, protective and deleterious effects, on rat hearts in a tightly time-course dependent, which may partially explain previous controversial effects of intermittent hypoxia or ischemia on cardiac damage or cardiac protection. After acute myocardial infarction (AMI), clinical trials had clearly shown the beneficial effects of early reperfusion within 12 hours, and possibly up to 24 hours [20], which can be explained by “open-artery hypothesis” [21]. Our findings in STIHH might provide a part of explanation for the “open-artery hypothesis” which stated that myocardial reperfusion, even if late for myocardial salvage, provides benefits and prevents adverse cardiac remodeling [21]. In the other side, our current findings and our previous Bcl2 family-related regulation [13] in LTIHH might provide a part of explanation for “post-infarction myocardial apoptosis” [2] and “post-infarction cardiac fibrosis” [22, 23]. Post-infarction myocardial apoptosis had been implicated as a cause of ongoing cell loss leading to cardiac failure and even the increased myocardial apoptosis occurred in the non-infracted remote myocardium [2]. Since

STIHH can promote myocardial cell survival, for training application, we may further proposed that cardiopulmonary training under short-term challenges of intermittent hypobaric hypoxia may be beneficial for cardiac function, which might partially contribute to the improvement of "training high-living low" [24]. Since LTIHH may aggravate myocardial cell death, for therapeutic and preventive application, we may further propose that there is a potential cardiac damage in people with “high mountain sickness” or in people ascending to high altitudes with a longer stay. Besides, it might be beneficial to block cardiac Fas signaling pathway when considering possible therapeutic agents to control the development of apoptosis and/or fibrosis-related cardiac diseases. Of course, further clinical studies are required to clarify whether apoptotic mechanisms under short-term versus long-term hypoxia plays a specific role in “open-artery hypothesis” versus “post-infarction myocardial apoptosis”, "training high-living low" versus “high mountain sickness”, and cardiac protection versus cardiac pathogenesis.

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References

[1]. Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res* 1998;**82**:1111-29.

[2]. Biondi-Zoccai GG, Abbate A, Vasaturo F, Scarpa S, Santini D, Leone AM, et al. Increased apoptosis in remote non-infarcted myocardium in multivessel coronary disease. *Int J Cardiol* 2004;**94**:105-10.

[3]. Narula J, Pandey P, Arbustini E, Haider N, Narula N, Kolodgie FD, et al. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy.[see comment]. *Proc Natl Acad Sci USA* 1999;**96**:8144-9.

[4]. Bishopric NH, Andreka P, Slepak T, Webster KA. Molecular mechanisms of apoptosis in the cardiac myocyte. *Curr Opin Pharmacol* 2001;**1**:141-50.

[5]. Reddy MB, Clark L. Iron, oxidative stress, and disease risk. *Nutr Rev* 2004;**62**:120-4.

[6]. Oudit GY, Trivieri MG, Khaper N, Husain T, Wilson GJ, Liu P, et al. Taurine supplementation reduces oxidative stress and improves cardiovascular function in an iron-overload murine model. *Circulation* 2004;**109**:1877-85.

[7]. Sorescu D, Griendling KK. Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. *Congest Heart Fail* 2002;**8**:132-40.

[8]. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000;**52**:11-34.

[9]. Stephanou A, Scarabelli TM, Brar BK, Nakanishi Y, Matsumura M, Knight RA, et al. Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1

transcription factor but not tyrosine 701. *J Biol Chem* 2001;**276**:28340-7.

[10]. Yaniv G, Shilkrut M, Lotan R, Berke G, Larisch S, Binah O. Hypoxia predisposes neonatal rat ventricular myocytes to apoptosis induced by activation of the Fas (CD95/Apo-1) receptor: Fas activation and apoptosis in hypoxic myocytes. *Cardiovasc Res* 2002;**54**:611-23.

[11]. Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, et al. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994;**75**:426-33.

[12]. Dong JW, Zhu HF, Zhu WZ, Ding HL, Ma TM, Zhou ZN. Intermittent hypoxia attenuates ischemia/reperfusion induced apoptosis in cardiac myocytes via regulating Bcl-2/Bax expression. *Cell Res* 2003;**13**:385-91.

[13]. Lee SD, Kuo WW, Wu CH, Lin YM, Lin JA, Lu MC, et al. Effects of short- and long-term hypobaric hypoxia on Bcl2 family in rat heart. *Int J Cardiol* 2005.

[14]. Date T, Mochizuki S, Belanger AJ, Yamakawa M, Luo Z, Vincent KA, et al. Differential effects of membrane and soluble Fas ligand on cardiomyocytes: role in ischemia/reperfusion injury. *J Mol Cell Cardiol* 2003;**35**:811-21.

[15]. Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, et al. Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. *Circulation* 2000;**102**:915-20.

[16]. Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, et al. Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. *Circ Res* 2002;**90**:745-8.

[17]. Chao W, Shen Y, Li L, Rosenzweig A. Importance of FADD signaling in serum deprivation- and hypoxia-induced cardiomyocyte apoptosis. *J Biol Chem* 2002;**277**:31639-45.

[18]. D'Armiento J. Matrix metalloproteinase disruption of the extracellular matrix and cardiac dysfunction. *Trends Cardiovasc Med* 2002;**12**:97-101.

[19]. Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodelling. *Nature* 2002;**415**:240-3.

[20]. Sadanandan S, Hochman JS. Early reperfusion, late reperfusion, and the open artery hypothesis: an overview. *Prog Cardiovasc Dis* 2000;**42**:397-404.

[21]. Abbate A, Biondi-Zoccai GG, Baldi A, Trani C, Biasucci LM, Vetrovec GW. The 'Open-Artery Hypothesis': new clinical and pathophysiologic insights. *Cardiology* 2003;**100**:196-206.

[22]. Takeshita K, Hayashi M, Iino S, Kondo T, Inden Y, Iwase M, et al. Increased expression of plasminogen activator inhibitor-1 in cardiomyocytes contributes to cardiac fibrosis after myocardial infarction. *Am J Pathol* 2004;**164**:449-56.

[23]. Yu CM, Tipoe GL, Wing-Hon Lai K, Lau CP. Effects of combination of angiotensin-converting enzyme inhibitor and angiotensin receptor antagonist on inflammatory cellular infiltration and myocardial interstitial fibrosis after acute myocardial infarction. *J Am Coll Cardiol* 2001;**38**:1207-15.

[24]. Hoppeler H, Vogt M. Hypoxia training for sea-level performance. Training high-living low. *Adv Exp Med Biol* 2001;**502**:61-73.

Table 1

Table 1 Effects of short-term (1 and 4 days) intermittent hypobaric hypoxia on rat hearts

	Normoxia	Hypoxia 1 day	Hypoxia 4 days
body weight (BW),g	390 ±22	394 ±10	370 ±35
whole heart weight (WHW),g	1.06 ±1.08	1.1 ±0.1	1.07 ±0.12
left ventricular weight (LVW),g	0.72 ±0.07	0.73 ±0.05	0.72 ±0.08
WHW / BW ($X10^3$)	2.72 ±0.22	2.79 ±0.26	2.89 ±0.41
LVW / BW ($X10^3$)	1.85 ±0.27	1.85 ±0.10	1.95 ±0.28

Values are means ± SEM (n=8). BW, body weight; WHW, whole heart weight; LVW, left ventricular weight. No significant difference between normobaric normoxia and hypobaric hypoxia 1 day or 4 days.

Table 2

Table 2 Effects of long-term intermittent (1 and 2 weeks) hypobaric hypoxia on rat hearts

	Normoxia	Hypoxia 1 weeks	Hypoxia 2 weeks
body weight (BW),g	416±16	406 ±27	404±24*
whole heart weight (WHW),g	1.13 ±0.05	1.15 ±0.07	1.25 ±0.1*
left ventricular weight (LVW),g	0.72 ±0.03	0.75 ±0.04	0.85 ±0.01*
WHW / BW ($X10^3$)	2.72 ±0.18	2.83 ±0.3	3.1±0.33*
LVW / BW ($X10^3$)	1.73 ±0.11	1.84 ±0.2	2.1 ±0.25*

Values are means ± SEM (n=8). BW, body weight; WHW, whole heart weight; LVW, left ventricular weight. * $P<0.05$ Significant difference between normobaric normoxia and hypobaric hypoxia 1 week or 2 week.

Figure Legend

Fig 1

Protocol diagram. Seven-two rats were randomized assigned into two groups, first, short-term intermittent hypobaric hypoxia (STIHH)- normbaric normoxia for 1 or 4 days (#1, n=12), hypobaric hypoxia for 1 day (#2, n=12), and 4 day (#3, n=12) and second, long-term intermittent hypobaric hypoxia (LTIHH)- normbaric normoxia for for 1 or 2 week (#4, normbaric normoxia, n=12), 1 week (#5, n=12), and 2 weeks (#6, n=12). N=normobaric normoxia (about 760 mm, 21% O_2 and 79% N_2); H=hypobaric hypoxia (380 mm, 12% O_2 and 88% N_2, for eight hours per day); n, number of rats.

Fig 2.

The protein products of Fas ligand and Fas receptor extracted from the left ventricles of rat hearts following two trials, (A) short-term intermittent hypobaric hypoxia i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 day group(H-1d, n=3), and hypobaric hypoxia 4 day group (H-4d, n=3); (B) long-term intermittent hypobaric hypoxia, i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 week group(H-1w, n=3), and hypobaric hypoxia 2 week group (H-2w, n=3), were measure by Western Blotting analysis. Bars represent the relative quantification on the basis of α-tubulin and indicate mean values±SD (n=9 in each group). * $P< 0.05$, significant differences between normobaric normoxia group and hypobaric hypoxia group.

Fig 3.

The mRNA expressions of tumor necrosis factor alpha (TNFα) extracted from the left ventricles of rat hearts following two trials, (A) short-term intermittent hypobaric hypoxia i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 day group(H-1d, n=3), and hypobaric hypoxia 4 day group (H-4d, n=3); (B) long-term intermittent hypobaric hypoxia, i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 week group (H-1w, n=3), and hypobaric hypoxia 2 week group(H-2w, n=3), were measure by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Bars represent the relative quantification on the basis glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and indicate mean values±SD (n=9 in each group). *P< 0.05, **P< 0.01, significant differences between normobaric normoxia group and hypobaric hypoxia group.

Fig 4

The protein products of Fas-associated death domain (FADD) extracted from the left ventricles of rat hearts following two trials, (A) short-term intermittent hypobaric hypoxia i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 day group(H-1d, n=3), and hypobaric hypoxia 4 day group (H-4d, n=3); (B) long-term intermittent hypobaric hypoxia, i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 week group(H-1w, n=3), and hypobaric hypoxia 2 week group(H-2w, n=3), were measure by Western Blotting analysis. Bars represent the relative quantification on the basis of α-tubulin and indicate mean values±SD (n=9 in each group). *P< 0.05, **P< 0.01, significant differences between normobaric normoxia group and hypobaric hypoxia group.

Fig 5

The protein products of caspase 8 extracted from the left ventricles of rat hearts following two trials, (A) short-term intermittent hypobaric hypoxia i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 day group(H-1d, n=3), and hypobaric hypoxia 4 day group (H-4d, n=3); (B) long-term intermittent hypobaric hypoxia, i.e. normobaric normoxia group (Nor, n=3), hypobaric hypoxia 1 week group(H-1w, n=3), and hypobaric hypoxia 2 week group(H-2w, n=3), were measure by Western Blotting analysis. Bars represent the relative quantification on the basis of α-tubulin and indicate mean values±SD (n=9 in each group). *P< 0.05, **P< 0.01, significant differences between normobaric normoxia group and hypobaric hypoxia group.

Fig 6

Histopathological analysis of cardiac tissue sections stained with (A)hematoxylin and eosin as well as stained with (B) Masson trichrome were measured after normobaric normoxia group (Normoxia), long-term intermittent hypobaric hypoxia for 1 week, (1w LTIHH), and LTIHH for 2 week (2w LTIHH). The images were magnified by 100 times.

Figures

Fig 1

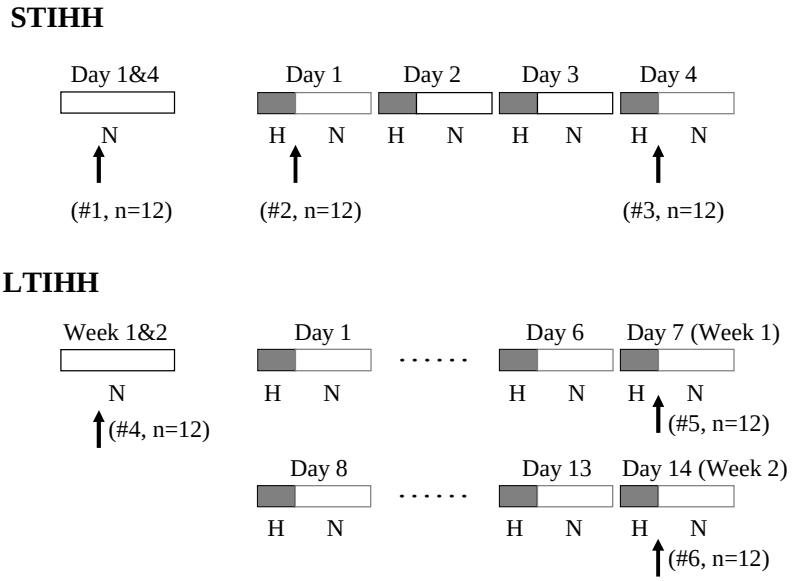


Fig 2

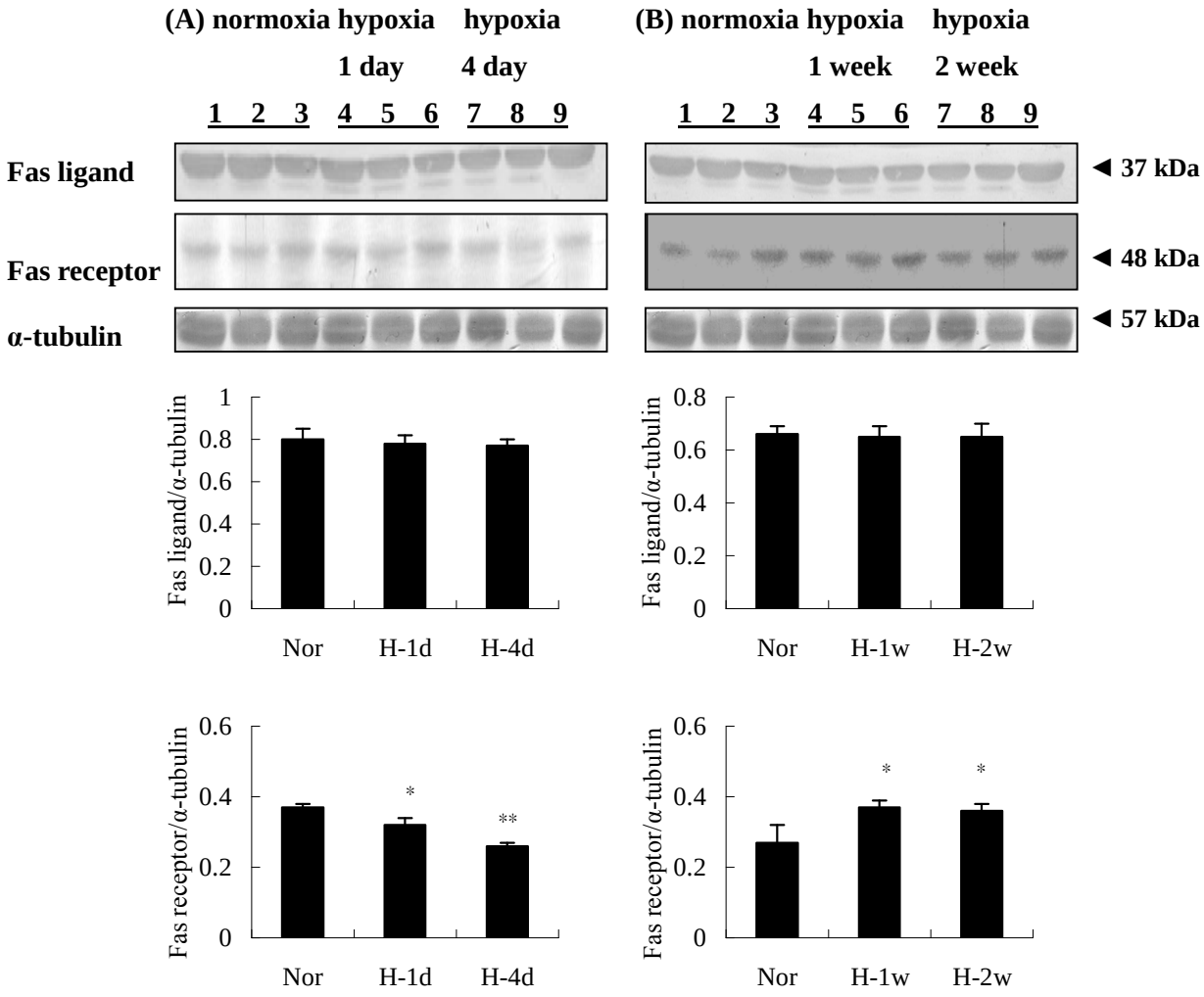

(A) normoxia hypoxia hypoxia
1 day 4 day
1 2 3 4 5 6 7 8 9
Fas ligand
Fas receptor
α-tubulin
◀ 37 kDa
◀ 48 kDa
◀ 57 kDa
Fas ligand/α-tubulin
Nor H-1d H-4d
(B) normoxia hypoxia hypoxia
1 week 2 week
1 2 3 4 5 6 7 8 9
Fas ligand/α-tubulin
Nor H-1w H-2w
Fas receptor/α-tubulin
Nor H-1d H-4d
Fas receptor/α-tubulin
Nor H-1w H-2w

Fig 3

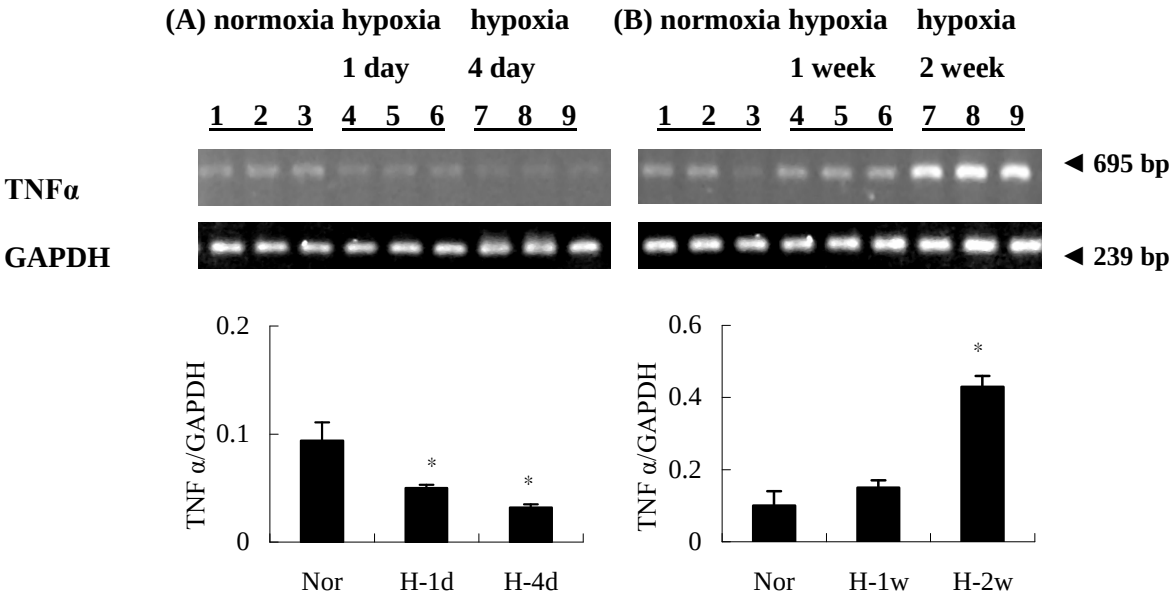

(A) normoxia hypoxia hypoxia
1 day 4 day
1 2 3 4 5 6 7 8 9
(B) normoxia hypoxia hypoxia
1 week 2 week
1 2 3 4 5 6 7 8 9
TNFα
GAPDH
◄ 695 bp
◄ 239 bp
TNF α/GAPDH
0.2
0.1
0
Nor
H-1d
H-4d
TNF α/GAPDH
0.6
0.4
0.2
0
Nor
H-1w
H-2w

Fig 4

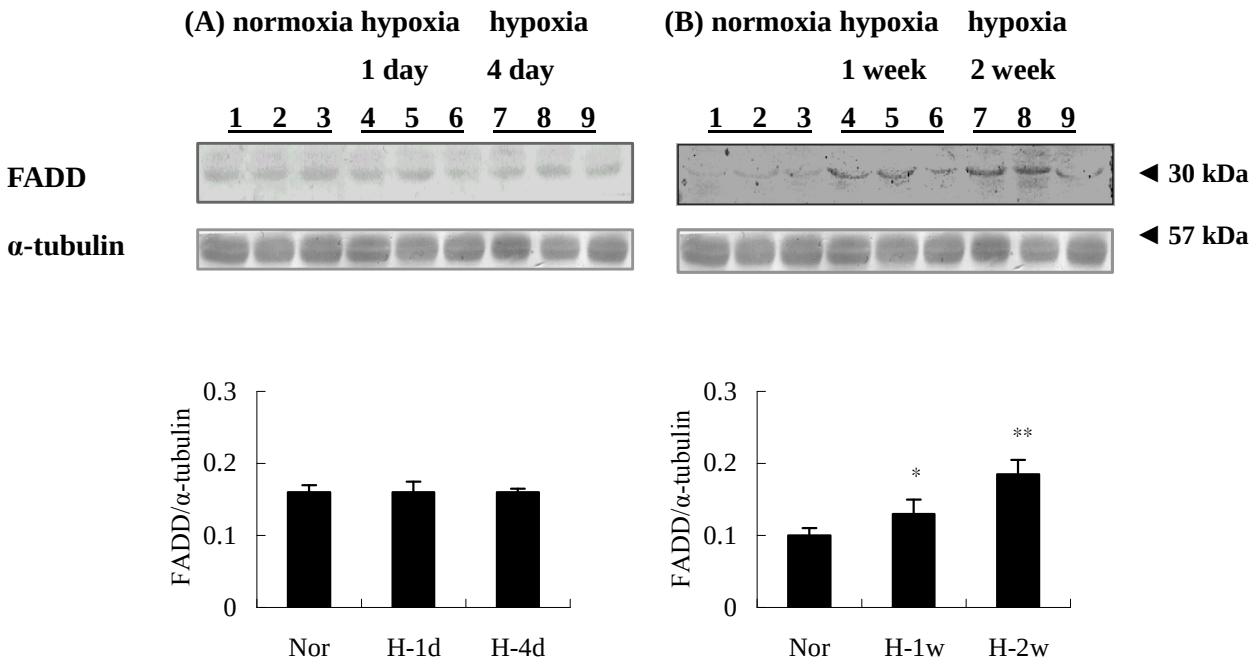

(A) normoxia hypoxia hypoxia
1 day 4 day
1 2 3 4 5 6 7 8 9
(B) normoxia hypoxia hypoxia
1 week 2 week
1 2 3 4 5 6 7 8 9
FADD
α-tubulin
◄ 30 kDa
◄ 57 kDa
FADD/α-tubulin
0.3
0.2
0.1
0
Nor
H-1d
H-4d
*
**
Nor
H-1w
H-2w

Fig 5

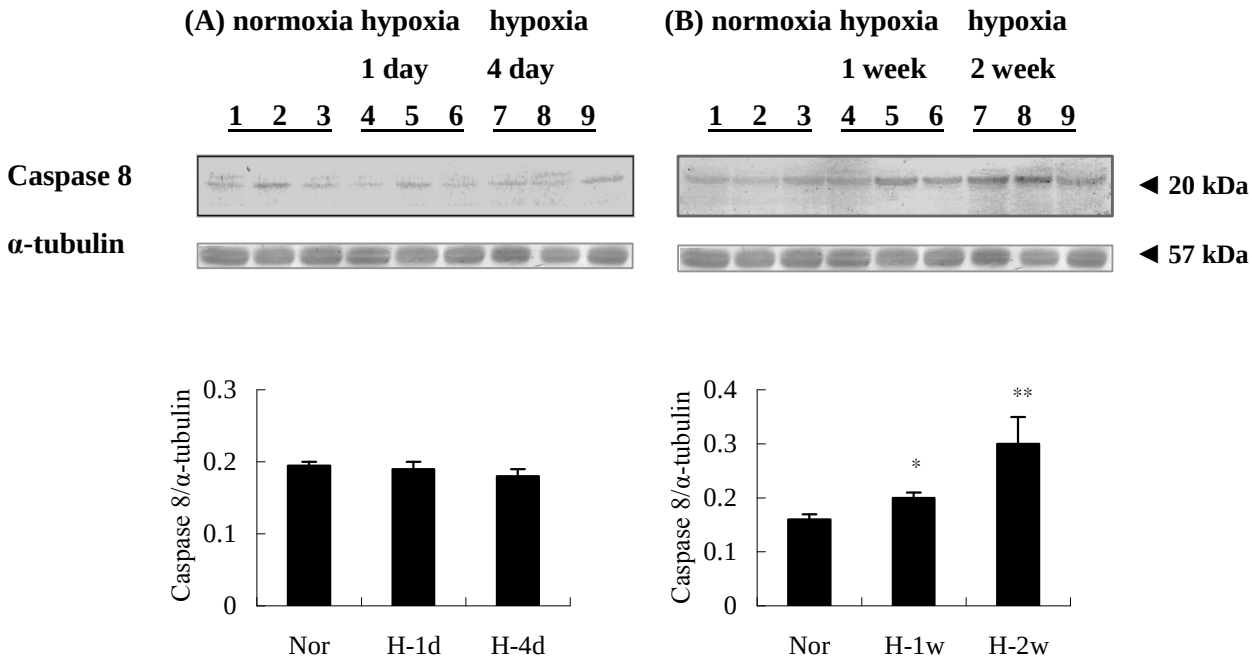
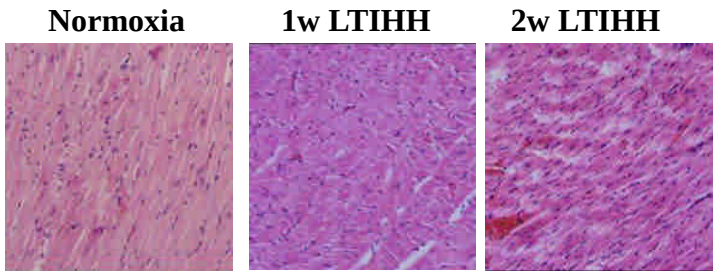

(A) normoxia hypoxia hypoxia
1 day 4 day
1 2 3 4 5 6 7 8 9
(B) normoxia hypoxia hypoxia
1 week 2 week
1 2 3 4 5 6 7 8 9
Caspase 8
α-tubulin
◄ 20 kDa
◄ 57 kDa
Caspase 8/α-tubulin
0.3
0.2
0.1
0
Nor
H-1d
H-4d
Caspase 8/α-tubulin
0.4
0.3
0.2
0.1
0
*
**
Nor
H-1w
H-2w

Fig 6

(A)



(B)