Intermittent Hypobaric Hypoxia Promotes Atherosclerotic Plaque Instability in ApoE-Deficient Mice

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Abstract

Jiang, Sihua, Feipeng Jin, De Li, Xingmei Zhang, Yun Yang, Dachun Yang, Kun Li, Yongjian Yang, and Shuangtao Ma. Intermittent hypobaric hypoxia promotes atherosclerotic plaque instability in ApoE-deficient mice. *High Alt Med Biol* 14:175–180, 2013.—**Aim:** Sudden cardiac death is one of the most frequent causes of death at high altitude. It has been reported that the intermittent normobaric hypoxia experienced by patients with obstructive sleep apnea may enhance the development of atherosclerosis. However, the effect of hypobaric hypoxia, which mimics the ambient air at high altitude, in the development of atherosclerosis has not been investigated. **Methods:** Twenty male ApoE-deficient mice, 8 weeks of age, were subjected to either control conditions or intermittent hypobaric hypoxia (IHH) for 8 weeks. Mice in the IHH group were exposed to a hypobaric chamber that replicated the hypobaric hypoxia conditions found at 4000 m altitude for 8 hours a day. **Results:** IHH-exposed mice did not significantly differ from control mice in plasma lipid levels, including triglyceride, total cholesterol, low-density lipoprotein, and high-density lipoprotein. The hematoxylin and eosin-stained sections of the aortic root showed similar plaque size between the groups. However, IHH-treated mice exhibited significantly decreased plaque collagen content, a feature of atherosclerotic plaque stability. Additionally, matrix metalloproteinase (MMP)-9 protein expression was significantly increased, whereas tissue inhibitor of MMP (TIMP)-2 expression was decreased after exposure to IHH. **Conclusion:** IHH may promote atherosclerotic plaque instability in ApoE-deficient mice by changing the balance of MMPs and TIMPs.

Key Words: altitude; atherosclerosis; hypobaric hypoxia; plaque instability

Introduction

ENVIRONMENTAL FACTORS CONTRIBUTE significantly to the increased rates of cardiovascular disease in both developed and developing countries (Brenn 1994; O'Toole et al., 2008). A large number of people live at a high altitude of more than 3000 meters around the world, especially in China (Wu et al., 2010). Moreover, there are also a number of people that ascend daily to these altitudes and are repeatedly exposed to hypoxia (Farias et al., 2006). Epidemiological studies have demonstrated that the risk of sudden cardiac death in populations with repetitive acute ascents to high altitude is much higher than that in populations living at low altitude (Burtcher and Ponchia 2010; Rimoldi et al., 2010). The possible mechanisms responsible for sudden deaths at high altitude include increases in adrenergic activity, abrupt changes in heart rate and blood pressure, and subsequent hemodynamic stress, in

part, due to physical exertion. The direct effects of repetitive acute ascents to high altitude on the risk for sudden death have not been elucidated. As high altitude may generally be a risk factor for acute coronary syndrome, the intermittent altitude exposure may promote atherosclerotic plaque instability.

High altitude settings are characterized by a peculiar environmental factor: hypoxia. It has been reported that intermittent normobaric hypoxia experienced by patients with obstructive sleep apnea may enhance the development of atherosclerosis. Previous studies have suggested that intermittent normobaric hypoxia accelerates atherosclerotic plaque growth in ApoE-deficient (ApoE^{-/-}) mice without affecting the plaque composition (Jun et al., 2010). Additionally, normobaric hypoxia was reported to cause atherosclerosis in C57BL/6 mice that were fed a high cholesterol diet (Savransky et al., 2007). However, the intermittent hypoxia that replicates obstructive sleep apnea may dramatically differ from a high

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altitude air environment. Thus, the role of hypobaric hypoxia, which mimics the ambient air at high altitude, in the development of atherosclerosis has not been elucidated. We hypothesized that intermittent hypobaric hypoxia (IHH) could affect atherosclerotic plaque growth and stability.

Collagen is an important component of the extracellular matrix in the atherosclerotic plaque. It has been shown that matrix collagen contributes to the integrity of the fibrous cap and the stability of the plaque (Nadkarni et al., 2009). The matrix metalloproteinases (MMPs), which are responsible for the proteolysis of matrix collagen, have emerged as important regulators of plaque instability (Beaudeux et al., 2004; Watanabe and Ikeda, 2004). The tissue inhibitors of metalloproteinase (TIMPs) are the endogenous inhibitors of MMPs. It was previously reported that hypoxia may affect several kinds of MMP in different tissues or cells. We therefore hypothesized that IHH could influence plaque stability by regulating the balance of MMPs and TIMPs.

In the present study, ApoE^{-/-} mice were fed a normal diet and exposed to control conditions or IHH for 8 weeks. Their lipid profiles, atherosclerotic plaque size, and surrogate markers of plaque stability were measured.

Materials and Methods

Animal care

Twenty 8-week-old male ApoE^{-/-} mice were purchased from the Model Animal Research Center of Nanjing Uni-

versity (Nanjing, China). The animals were housed under a 12 h/12 h day/night cycle and standard conditions of temperature (22 ± 1°C) and humidity (50%–60%) with *ad libitum* food and water. The mice were fed a normal chow diet. The experimental procedures were approved by the Hospital Animal Care and Use Committee.

Hypobaric hypoxia

A hypobaric chamber with pneumatic pumps was used to establish the hypobaric hypoxia condition replicating an altitude of 4000 meters (Pio₂ 12.7 kPa). Mice were randomly placed in control (*n* = 10) and IHH (*n* = 10) groups. Mice in the IHH group were placed into hypobaric chambers for 8 h (9:30 ~ 17:30) a day. The target air pressure was reached 2 min after initiating the experiments. Mice in the control group were placed alongside the chamber to control for the effect of pump noise on the animals. The administration period lasted 8 weeks.

Serum lipids

Fasting blood samples were obtained and triglycerides (TG), total cholesterol (TC), LDL cholesterol (LDL-C), and HDL cholesterol (HDL-C) were measured by colorimetric assays using a commercially available kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

FIG. 1. The effects of IHH on the metabolic character of ApoE^{-/-} mice. The daily food intake (A), body weight (B), triglyceride (C), total cholesterol (D), LDL-cholesterol (E), and HDL-cholesterol (F) of ApoE^{-/-} mice from the control (open bars) and IHH (solid bars) groups after 8 weeks of treatment. Data are expressed as mean ± SEM. *n* = 7 mice per group; ***p* < 0.01 compared with the control group. HDL, high-density lipoprotein; IHH, intermittent hypobaric hypoxia; LDL, low-density lipoprotein.

FIG. 2. The effects of IHH on atherosclerotic plaque size in ApoE^{-/-} mice. **(A)** Representative photographs of thoraco-abdominal lesions in ApoE^{-/-} mice exposed to control and IHH conditions. Lesions are stained with Oil Red O and shown at 10X magnification. **(B)** The plaque size was measured as a percentage of the total aortic surface. **(C)** Representative photographs of aortic root lesions stained with hematoxylin and eosin. **(D)** The plaque size of the aortic root is expressed as μm^2 . Data are expressed as mean $\pm$ SEM. $n=5$ mice per group; IHH, intermittent hypobaric hypoxia.

Atherosclerosis analysis

Atherosclerotic lesions of the thoraco-abdominal aorta were analyzed by Oil-red-O staining. Atherosclerotic lesions in aortic roots were examined in cross-sections of aortic origin. Six consecutive 5 μm thick sections were cut from the aorta where the valve cusp was visible. The sections were stained with hematoxylin (90 s) and eosin (2 s) and photographed

FIG. 3. The effects of IHH on plaque instability in ApoE^{-/-} mice. **(A)** Representative photographs of aortic lesions stained with Masson's Trichrome. **(B)** Collagen deposition was expressed as the percentage of positively stained area. Data are expressed as mean $\pm$ SEM. $n=5$ mice per group; ** $p<0.01$ compared with the control group. IHH, intermittent hypobaric hypoxia.

on an Olympus BX41 microscope (Olympus Corp., Tokyo, Japan). The atherosclerotic lesion area was quantified using Nikon NIS-Elements Research Software (Ma et al., 2011). The average plaque area was used for statistical analysis.

Collagen analysis

Paraffin-embedded aortic sinus sections were stained with Masson's Trichrome using a fast Masson dye kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The percentage of positively stained area was calculated using Nikon NIS-Elements research software.

Immunohistochemistry

Thoracic aortas were fixed in 4% paraformaldehyde for 12 h, embedded in paraffin, and then cut into sections (5 μm). The sections were incubated with anti-MMP-2, anti-MMP-3, anti-MMP-9, anti-MMP-14, anti-TIMP-1, and anti-TIMP-2 antibodies (diluted in 1:50 dilution, Boster Bioengineering Co., Wuhan, China). Specific binding was detected with biotinylated goat anti-rabbit IgG secondary antibody-horseradish peroxidase complexes using an ABC kit (Boster Bioengineering Co.). The antigen-antibody complex was subsequently visualized with 3', 3'-diaminobenzidine solution. Sections were viewed under a light microscope. The proportion of the positively stained area was calculated using Nikon NIS-Elements research software.

Statistical analysis

Continuous data are presented as a mean $\pm$ SEM. Comparisons between groups were determined by a one-way ANOVA with a post-hoc Student *t*-test (SPSS Inc., Chicago, IL). Probabilities of $p < 0.05$ were considered statistically significant.

Results

Food intake, body weight, and lipid profiles

The daily food intake for each mouse was recorded during the course of the study. The amount of food consumed was similar between the control and IHH groups (Fig. 1A). The baseline body weight was similar between the two groups. At the end of the experiment, mice exposed to IHH had lower body weight than the controls ($p < 0.01$) (Fig. 1B). Exposure to IHH did not affect the plasma lipid profiles, including TG, TC, LDL-C, and HDL-C (Fig. 1C–F).

Atherosclerotic plaque size

The atherosclerotic plaque size in the thoraco-abdominal aorta was similar between the control and IHH groups (Fig. 2A, B). Additionally, there was no difference in the mean plaque size within the aortic root between the two groups (Fig. 2C, D).

Plaque instability

As hypothesized, collagen deposition was significantly decreased in the IHH group compared to the control group ($p < 0.01$) (Fig. 3A, B).

MMP/TIMP expression

The expression of MMP-2, MMP-3, MMP-9, MMP-14, TIMP-1, and TIMP-2 protein in the thoracic aorta was measured by immunohistochemical staining. MMP-3 and TIMP-1 were not obviously visible after staining. Exposure to IHH significantly increased the expression of MMP-9 ($p < 0.01$), decreased the expression of TIMP-2 ($p < 0.01$), and had no significant effect on MMP-2 and MMP-14 in aortic sections (Fig. 4A, B).

Discussion

Hypoxia has been implicated in the development of atherosclerosis (Hulten and Levin, 2009; Mayr et al., 2008). Most previous studies have focused primarily on the harmful effects of normobaric hypoxia on patients with obstructive sleep apnea (Arnaud et al., 2009; Drager et al., 2005; Levy et al., 2009). Chronic intermittent hypoxia causes atherosclerotic lesions in C57BL/6 wild-type mice, which are usually resistant to atherosclerosis (Savransky et al., 2007). Additionally, intermittent hypoxia accelerates atherosclerotic plaque growth in ApoE^{-/-} mice (Jun et al., 2010; Nakano et al., 2005). Although the aforementioned conditions dramatically differ from the air environment at high altitude, it is reasonable that exposure to IHH could affect the development of atherosclerosis. Unfortunately, the present study demonstrated that IHH had no effect on the plaque size in ApoE^{-/-} mice. It is herein proposed that hypobaric conditions could attenuate the hypoxia-induced promotion of atherosclerosis.

There is a growing body of evidence that the ascent to high altitude is associated with a high risk of sudden cardiac death

FIG. 4. The effects of IHH on MMP-2/-9/-14 and TIMP-2 expression in aortas from ApoE^{-/-} mice. **(A)** Representative immunohistochemical stains showing the expression of MMP-2, MMP-9, MMP-14, and TIMP-1 in the aorta. **(B)** The levels of protein expression were expressed as the percentage of positively stained area. Data are expressed as mean $\pm$ SEM. $n = 5$ mice per group; ** $p < 0.01$ compared with the control group. IHH, intermittent hypobaric hypoxia; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase.

(Bartsch and Gibbs 2007; Bartscher and Ponchia 2010; Rimoldi et al., 2010). However, a causal link between altitude environment and sudden death has not been established. The main finding of this study was that 8-week IHH exposure resulted in plaque instability, as expressed by decreased collagen deposition. This result indicates that a high altitude environment mainly characterized as IHH may induce acute coronary syndrome and sudden cardiac death by increasing the vulnerability of the atherosclerotic plaque. A previous

study demonstrated that hypoxia alone could not affect plaque composition, including fibrotic caps and collagen deposition (Jun et al., 2010). Taken together, the effects on plaque instability may mostly be attributed to hypobaric conditions.

Chronic hypoxia induces adaptive metabolic changes in several different types of cells (Lodi et al., 2011; Plunkett et al., 1996). We found that exposure to IHH significantly decreased body weights of mice but did not affect the food intake of mice. The observed weight loss was consistent with previous studies (Jun et al., 2010; Savransky et al., 2007). The reduction in body weight may be attributed to the metabolic switch caused by hypoxia. As body weight control has a protective role in atherosclerosis, this might counteract hypoxia-induced plaque growth. In prior studies of mice exposed to normobaric hypoxia, atherogenic lipid profiles were significantly enhanced (Arnaud et al., 2009; Li et al., 2007; Nakano et al., 2005; Roche et al., 2009; Savransky et al., 2008). However, the present study demonstrated no effect on serum lipids after 8 weeks under IHH conditions. We hypothesize that the IHH-induced decrease in body weight may offset the changes in lipids.

Elevated blood pressure is an important factor in the development of atherosclerosis. Previous studies have demonstrated that hypoxia has many effects on blood pressure in rodents, including acute decreases in blood pressure (Campen et al., 2004) superimposed on chronic increases in blood pressure (Fletcher, 2000). However, another study showed that chronic intermittent hypoxia did not affect the blood pressure of Chilean mine workers (Farias et al., 2006). The present study demonstrated that the tail-cuff blood pressure was similar between the two groups (data not shown). However, the tail-cuff blood pressure was measured only once at the end of the present study. Moreover, the ambulatory blood pressure was not evaluated in the present study, and whether plaque instability is attributed to changes in blood pressure remains unclear.

Mechanisms by which hypoxia causes plaque instability have not been well established (Martin et al., 1991). MMPs and TIMPs have been viewed as the regulators of plaque instability (Murillo et al., 2009). We found that the plaque instability induced by IHH was concomitant with the increased MMP-9 and decreased TIMP-2 expression. A previous study demonstrated that hypoxia elevated the level of MMP-9 in cultured microvascular endothelial cells from the human brain (Kolev et al., 2003). Moreover, hypoxia lowers TIMP-2 protein secretion by human endothelial cells (Lahat et al., 2011). Taken together, the increased MMP-9 levels and decreased TIMP-2 levels induced by hypoxia may stimulate the degradation of collagen and consequently cause plaque instability. The results of the present study indicate that the imbalance of MMP and TIMP might be responsible for the linkage between IHH and plaque vulnerability. Therefore, restoring the balance between MMPs and TIMPs may be a novel strategy to prevent hypobaric hypoxia-induced plaque instability.

In conclusion, the present results demonstrate that exposure to IHH promotes atherosclerotic plaque instability without affecting plaque size in ApoE^{-/-} mice. Moreover, the IHH-induced vulnerability to plaques is associated with the imbalance between MMP-9 and TIMP-2. The current study also provides a model of hypobaric hypoxia for the study of atherosclerosis at high altitude. The present findings may contribute to mechanistic insights into altitude-associated sudden cardiac death.

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References

- Arnaud C, Dematteis M, Pepin JL, Baguet JP, and Levy P. (2009). Obstructive sleep apnea, immuno-inflammation, and atherosclerosis. *Semin Immunopathol* 31:113–125.
- Bartsch P, and Gibbs JS. (2007). Effect of altitude on the heart and the lungs. *Circulation* 116:2191–2202.
- Beaudeux JL, Giral P, Bruckert E, Foglietti MJ, and Chapman MJ. (2004). Matrix metalloproteinases, inflammation and atherosclerosis: Therapeutic perspectives. *Clin Chem Lab Med* 42: 121–131.
- Brenn T. (1994). Genetic and environmental effects on coronary heart disease risk factors in northern Norway. The cardiovascular disease study in Finnmark. *Ann Hum Genet* 58: 369–379.
- Burtscher M, and Ponchia A. (2010). The risk of cardiovascular events during leisure time activities at altitude. *Prog Cardiovasc Dis* 52:507–511.
- Campen MJ, Tagaito Y, Li J, et al. (2004). Phenotypic variation in cardiovascular responses to acute hypoxic and hypercapnic exposure in mice. *Physiol Genom* 20:15–20.
- Drager LF, Bortolotto LA, Lorenzi MC, Figueiredo AC, Krieger EM, and Lorenzi-Filho G. (2005). Early signs of atherosclerosis in obstructive sleep apnea. *Am J Respir Crit Care Med* 172: 613–618.
- Farias JG, Osorio J, Soto G, Brito J, Siques P, and Reyes JG. (2006). Sustained acclimatization in Chilean mine workers subjected to chronic intermittent hypoxia. *High Alt Med Biol* 7:302–306.
- Fletcher EC. (2000). Effect of episodic hypoxia on sympathetic activity and blood pressure. *Respir Physiol* 119:189–197.
- Hulten LM, and Levin M. (2009). The role of hypoxia in atherosclerosis. *Curr Opin Lipidol* 20:409–414.
- Jun J, Reinke C, Bedja D, et al. (2010). Effect of intermittent hypoxia on atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 209:381–386.
- Kolev K, Skopal J, Simon L, Csonka E, Machovich R, and Nagy Z. (2003). Matrix metalloproteinase-9 expression in post-hypoxic human brain capillary endothelial cells: H₂O₂ as a trigger and NF-kappaB as a signal transducer. *Thromb Haemost* 90:528–537.
- Lahat N, Bitterman H, Engelmayer-Goren M, Rosenzweig D, Weiss-Cerem L, and Rahat MA. (2011). Reduced TIMP-2 in hypoxia enhances angiogenesis. *Am J Physiol Cell Physiol* 300:C557–566.
- Levy P, Pepin JL, Arnaud C, Baguet JP, Dematteis M, and Mach F. (2009). Obstructive sleep apnea and atherosclerosis. *Prog Cardiovasc Dis* 51:400–410.
- Li J, Savransky V, Nanayakkara A, Smith PL, O'Donnell CP, and Polotsky VY. (2007). Hyperlipidemia and lipid peroxidation are dependent on the severity of chronic intermittent hypoxia. *J Appl Physiol* 102:557–563.

- Lodi A, Tiziani S, Khanim FL, Drayson MT, Gunther UL, Bunce CM, and Viant MR. (2011). Hypoxia triggers major metabolic changes in AML cells without altering indomethacin-induced TCA cycle deregulation. *ACS Chem Biol* 6:169–175.
- Ma S, Yang D, Li D, Tang B, and Yang Y. (2011). Oleic acid induces smooth muscle foam cell formation and enhances atherosclerotic lesion development via CD36. *Lipids Health Dis* 10:53.
- Martin JF, Booth RF, and Moncada S. (1991). Arterial wall hypoxia following thrombosis of the vasa vasorum is an initial lesion in atherosclerosis. *Eur J Clin Invest* 21:355–359.
- Mayr M, Sidibe A, and Zampetaki A. (2008). The paradox of hypoxic and oxidative stress in atherosclerosis. *J Am Coll Cardiol* 51:1266–1267.
- Murillo CA, Woodside KJ, Guo Q, Zhang S, O'Connor KL, and Hunter GC. (2009). Integrin and matrix metalloproteinase expression in human carotid plaque. *J Surg Res* 155:157–164.
- Nadkarni SK, Bouma BE, de Boer J, and Tearney GJ. (2009). Evaluation of collagen in atherosclerotic plaques: The use of two coherent laser-based imaging methods. *Lasers Med Sci* 24:439–445.
- Nakano D, Hayashi T, Tazawa N, et al. (2005). Chronic hypoxia accelerates the progression of atherosclerosis in apolipoprotein E-knockout mice. *Hypertens Res* 28:837–845.
- O'Toole TE, Conklin DJ, and Bhatnagar A. (2008). Environmental risk factors for heart disease. *Rev Environ Health* 23:167–202.
- Plunkett MD, Hendry PJ, Anstadt MP, et al. (1996). Chronic hypoxia induces adaptive metabolic changes in neonatal myocardium. *J Thorac Cardiovasc Surg* 112:8–13.
- Rimoldi SF, Sartori C, Seiler C, et al. (2010). High-altitude exposure in patients with cardiovascular disease: Risk assessment and practical recommendations. *Prog Cardiovasc Dis* 52:512–524.
- Roche F, Sforza E, Pichot V, et al. (2009). Obstructive sleep apnoea/hypopnea influences high-density lipoprotein cholesterol in the elderly. *Sleep Med* 10:882–886.
- Savransky V, Jun J, Li J, et al. (2008). Dyslipidemia and atherosclerosis induced by chronic intermittent hypoxia are attenuated by deficiency of stearyl coenzyme A desaturase. *Circ Res* 103:1173–1180.
- Savransky V, Nanayakkara A, Li J, et al. (2007). Chronic intermittent hypoxia induces atherosclerosis. *Am J Respir Crit Care Med* 175:1290–1297.
- Watanabe N, and Ikeda U. (2004). Matrix metalloproteinases and atherosclerosis. *Curr Atheroscler Rep* 6:112–120.
- Wu TY, Ding SQ, Zhang SL, et al. (2010). Altitude illness in Qinghai-Tibet railroad passengers. *High Alt Med Biol* 11:189–198.

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