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Original Article

Acute exposure to hypobaric hypoxia upregulates the expression of hypoxia-inducible factor-1α and vascular endothelial growth factor

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Abstract: Hypoxia is a stress factor frequently encountered during flight and a common cause of tissue and cell injury experienced by in-flight crew. Effects of hypoxia on the body can vary depending on the duration and severity of hypoxic exposure. As symptoms can differ among individuals, the measures taken to address hypoxia can be greatly improved by understanding its effects. It is critically important for pilots, cabin crew, and in-flight medical professionals to familiarize themselves with hypoxia and the factors that affect its presentation. In this study, we investigated the effect of hypoxia on the expression of vascular endothelial growth factor (VEGF) in mice using a hypoxic-exposure model. Experimental animals were placed in a hypobaric chamber at 8,000 ft for 1 h (n=5), 3 h (n=5), or 6 h (n=5). Immediately after hypoxic exposure, protein concentration of VEGF and mRNA levels of hypoxia-inducible factor-1α (HIF-1α) and VEGF were analyzed in serum and liver tissue homogenates. Exposure to hypobaric hypoxia significantly upregulated the expression of both HIF-1α and VEGF mRNA, but not hepatic VEGF mRNA. Our data indicate that acute exposure to hypobaric hypoxia upregulates serum mRNA levels of HIF-1α and VEGF in mice, and that the liver is less likely to be the source of elevated serum VEGF mRNA. In contrast, serum VEGF protein level may be regulated by other factors. Further investigations to confirm or disprove our preliminary results are required.

Keywords: Acute hypobaric hypoxia, hypoxia-inducible factor-1α, vascular endothelial growth factor

Introduction

Hypoxia is one of the most frequently encountered stress factors, and it is often implicated as a common cause of tissue and cell injury. Recent studies have indicated that the effects of hypoxia on the body can vary depending on the duration, severity, and frequency of hypoxic exposure [1]. Individuals may be exposed to acute or chronic hypoxia during their lifetime, and previous studies have reported the effects of hypoxia on the organs and tissues. Hypoxia can be observed in both physiological and pathophysiological conditions such as those encountered during severe exercise, air travel, obstructive sleep apnea, exposure to high altitude, and various respiratory diseases [1].

The aerospace environment has negative effects on body functions, resulting in the malfunction of vital organs. Flight surgeons continually seek to determine and understand the physiological responses of pilots to hypoxia and the pathophysiological mechanisms involved. Advances in aircraft design have made it possible to fly at increasingly higher altitudes. However, the possibility of experiencing hypoxia still exists, not only for pilots and cabin crew, but also for patients being transported by military or civilian airlines [2]. As symptoms of hypoxia can vary among individuals, the measures taken to address hypoxia can be greatly improved by understanding its effects. It is critically important for pilots, cabin crew, and medical professionals to become familiar with hypoxia and the factors that affect its presentation.

Vascular endothelial growth factor (VEGF), previously known as vascular permeability factor,

is a potent endothelial cell-specific mitogen involved in the regulation of vascular permeability and angiogenesis [3]. When administered to endothelial cells in culture media, VEGF induces several of the steps involved in formation of new blood vessels, including proliferation and migration of endothelial cells and elicitation of *de novo* blood vessel formation. VEGF also triggers the production of matrix metalloproteinases, which are required for breakdown of the basement membrane and invasion of blood vessels into the surrounding stroma [4].

Hypoxia stimulates the expression of both VEGF and its receptors in human endothelial cells [5]. Xu et al. [6] demonstrated that VEGF expression in the brain is strongly upregulated by alveolar hypoxia, indicating the association of VEGF with the pathogenesis of high-altitude cerebral edema. Christou et al. [7] also observed that hypoxia significantly upregulated gene expression of VEGF and its receptors in rat lungs. Similarly, a previous human study by Walter et al. [8] reported that serum VEGF concentration significantly increased in mountain climbers 24 h after they reached an altitude of 4,559 m (14,957 ft).

Commercial aircraft pressurization can provide a "shirt-sleeve environment" within the cabin, with an equivalent altitude of 8,000 ft, while the aircraft can operate up to approximately 40,000 ft. In contrast, military combat aircrafts are pressurized to a lower degree than commercial or noncombat planes, because they are more likely to sustain damage that can result in loss of pressurization [9]. At an altitude of 8,000 ft, the ambient partial pressure of oxygen is 118.29 mmHg and alveolar oxygen tension is 68.9 mmHg, which are 26% and 34% lower than those at sea level, respectively. Though an altitude of 8,000 ft falls within the physiologically efficient zone, one can experience shortness of breath, fatigue, dizziness, and headache with prolonged exposure [10].

In this study, we used an animal hypoxic-exposure model to investigate whether acute exposure to hypobaric hypoxia alters the mRNA expression levels of hypoxia-inducible factor alpha (HIF-1α) and VEGF as well as VEGF protein concentration. Further, the source of elevated serum VEGF mRNA was determined by analyzing levels of hepatic VEGF mRNA. We observed that acute hypoxic exposure significantly upregulates serum mRNA levels of HIF-1α and VEGF, suggesting a role of hypoxia in regulation of the VEGF signaling pathway. The expression pattern of hepatic VEGF mRNA did not correlate with change in serum VEGF mRNA level.

Materials and methods

Experimental animals and hypoxia exposure

An experimental scheme is shown in **Figure 1**. Twenty-five 8-yr-old C57BL/6 mice were used. Throughout the experimental period, the animals were fed standard laboratory mouse chow and provided free access to water. The animals were randomly divided into hypoxic and control (normoxic) groups. To achieve hypoxia, mice were placed in an altitude chamber at a barometric pressure of 570 mmHg, corresponding to an altitude of 2,438 m (approximately 8,000 ft). The temperature and moisture of the chamber were maintained at 20°C to 24°C and 45%, respectively. The animals in the hypoxic groups (n=5 each) were exposed to these conditions for 1, 3, or 6 h. The mice in the control group (n=5) were housed in the same environment as the hypoxic group, with access to food and water *ad libitum*, except that they were allowed to breathe normal room air. Later, the mice were laparotomized via a midline incision to collect tissue samples. Liver tissue was immediately frozen in liquid nitrogen at -80°C until reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed. Whole blood was collected by cardiac puncture and centrifuged at 3,000 rpm (approximately 1,000 *g*) for 10 min to obtain serum. The care of animals and experimental procedures were in accordance with guidelines established by the National Institutes of Health. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Republic of Korea Aerospace Medical Center (Cheongju, Chungcheongbuk-do, Republic of Korea; Protocol No.: ASMC-14-IACUC-001).

mRNA expression of HIF-1α and VEGF

Real-time RT-PCR was carried out for HIF-1α and VEGF to quantify gene expression of HIF-1α and VEGF under hypoxia. Quantitative real-time RT-PCR was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with a C1000

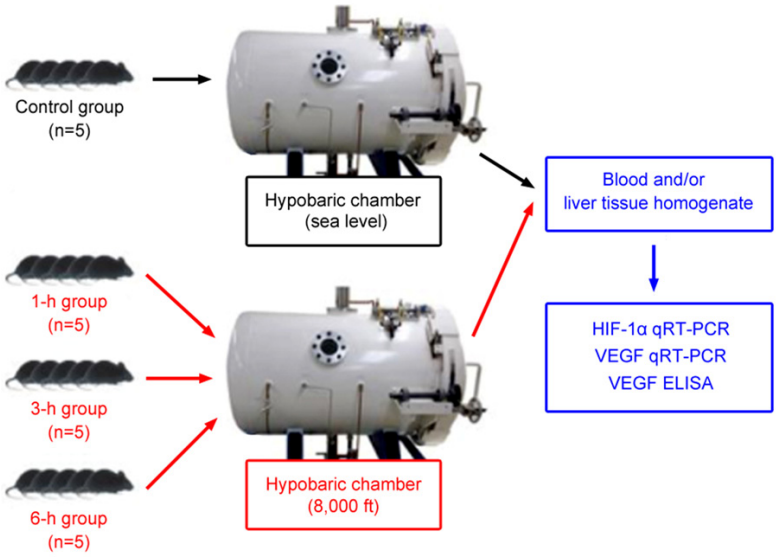

Figure 1. Experimental scheme of hypoxic exposure. Experimental animals (n=5 each) were exposed to hypobaric hypoxia (8,000 ft) for 1, 3, or 6 h. Serum and hepatic HIF-1α and VEGF mRNA levels were measured using quantitative real-time RT-PCR, and serum VEGF concentration was analyzed using ELISA.

Thermal Cycler (Bio-Rad Laboratories). Total RNA from serum and liver tissue was isolated using the NucleoSpin RNA II extraction kit (Macherey-Nagel, Dueren, Germany), according to the manufacturer's instructions. cDNA synthesis was performed using the ReverTra Ace-α-reverse-transcriptase kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Standard cDNA was quantified photometrically. Reverse-transcribed cDNA was used for real-time RT-PCR using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories). PCR was initiated with a denaturing step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec, 58°C for 10 sec, and 72°C for 20 sec. A melting curve analysis from 65°C to 95°C was performed following each RT-PCR in order to test for the presence of primer dimers. When primer-dimer formation was detected, the PCR was repeated using a fresh aliquot of cDNA. Each measurement was repeated three times, and the values were used to calculate the ratios of HIF-1α/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and VEGF/GAPDH, with a value of 1.0 used as the control (calibrator).

Measurement of serum VEGF concentration

Serum VEGF concentration was determined by enzyme-linked immunosorbent assay (ELISA) using a VEGF (R&D Systems, Quantikine ELISA, MN) assay kit, according to the manufacturer's instructions. VEGF level was determined by measuring optical density using a microplate reader (Bio-Rad Laboratories) at 450 nm.

Statistical analysis

All values are provided as mean ± standard error. Differences in the normalized mRNA ratio and protein concentration between groups were assessed using the Kruskal-Wallis test (SPSS ver. 18.0 software; IBM SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

Results

Significant increases in HIF-1α mRNA expression in the serum of hypoxic mice were observed in the 3- and 6-h exposure groups (**Figure 2A**). Quantitative real-time RT-PCR analysis of HIF-1α mRNA revealed 1.60-, 2.67-, and 1.88-fold increases in the amount of mRNA in the 1-, 3-, and 6-h exposure groups, respectively.

Serum VEGF mRNA level showed a trend similar to that of HIF-1α mRNA expression (**Figure 2B**). Significant increases in VEGF mRNA expression in the serum of hypoxic mice were observed in the 1- and 3-h exposure groups. Quantitative analysis revealed 1.69-, 3.17-, and 1.36-fold increases in the amount of VEGF mRNA in the 1-, 3-, and 6-h exposure groups, respectively. To determine the source of elevated serum VEGF mRNA, hepatic VEGF mRNA level was also analyzed. However, the time course of changes in hepatic VEGF mRNA level was different from that of serum mRNA level (**Figure 2C**); a significant upregulation of hepatic VEGF mRNA was observed only in the 6-h exposure group (1.80-fold compared to the control group). The 1-h exposure group displayed a slight increase in the amount of mRNA compared to the control group, whereas the 3-h exposure group showed a decrease below the

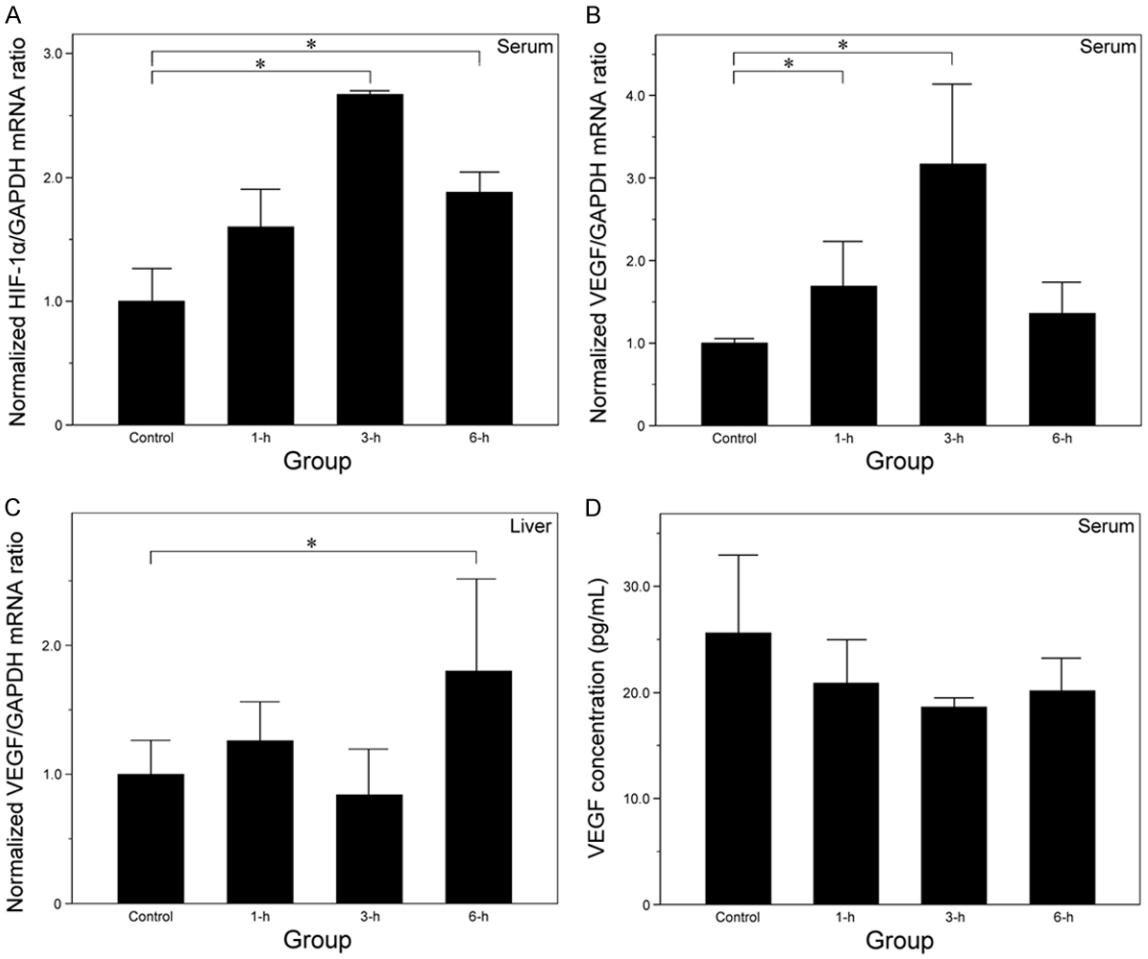

Figure 2. Effect of exposure to hypoxia on the expression of HIF-1α and VEGF. (A and B) Time course of expression of serum HIF-1α mRNA and VEGF mRNA. Significant increases in the amount of (A) HIF-1α and (B) VEGF mRNA were observed in hypoxic mice. (C) Time course of expression of hepatic VEGF mRNA. The 6-h exposure group showed a significantly higher mRNA level than the control group, whereas the other two groups did not show a significant difference in VEGF mRNA level compared to the control group. (D) Time course of expression of serum VEGF concentration. Compared to the control group, the three experimental groups displayed lower VEGF levels, although the differences between the control group and each experimental group were not significant.

basal level found in the control group. These results indicate that the liver is unlikely to be involved in the upregulation of serum VEGF mRNA and protein expression following hypoxic exposure. Similarly, the time course of alterations in serum VEGF level was different from that of VEGF mRNA level. As shown in **Figure 2D**, The mean serum VEGF level of the control group was 25.58±7.96 pg/mL. After hypoxic exposure, the levels decreased, although the difference was insignificant; the mean serum VEGF concentrations of the 1-, 3-, and 6-h exposure groups were 20.87±4.06, 18.61±1.09, and 20.15±3.33 pg/mL, respectively. These findings suggest that serum VEGF protein level is regulated by other factors. None of the mice displayed remarkable changes in behavior during or after exposure to hypoxia.

Discussion

We observed that mRNA expression levels of HIF-1α and VEGF increased significantly under hypobaric hypoxia. HIF-1α and VEGF mRNA levels were higher in the 1- and 3-h exposure groups than in the control group. Additionally, mRNA levels increased to a greater extent in the 3-h exposure group than in the 1-h exposure group, although the difference was not statistically significant. The 6-h exposure group

showed a lower normalized ratio than the 3-h exposure group; however, it was still higher than that in the control group. These results suggest that exposure to hypobaric hypoxia triggers hypoxia-induced cellular signaling cascades, including HIF-1α and VEGF signaling pathways. This finding indicates the possibility that at least 3 to 6 h is needed for physiologic adaptation to hypoxia at an altitude of 8,000 ft.

Results of previous studies investigating the effects of hypoxic exposure on serum VEGF concentration are controversial. Schobersberger et al. [11] investigated the effects of exhaustive long-lasting exercise at moderate altitude on the time course of changes in serum VEGF level, where 13 well-trained runners who participated in the Swiss Alpine Marathon of Davos (distance, 67,000 m; altitude difference, 2,300 m) were observed. In their study, serum VEGF increased significantly, immediately after the run, and was maintained at a significantly high level (up to 2.4-fold higher) until 5 d post-exercise. In contrast, Maloney et al. [12] found that high-altitude VEGF level was not significantly higher than that measured at sea level in mountaineers at high altitude (14,200 ft). In the present study, serum VEGF concentration was not significantly altered following hypoxic exposure but did decrease slightly. This result is consistent with that of Gunga et al. [13], who investigated the time course of changes in serum VEGF concentration during a high-altitude marathon run. They showed that prolonged physical stress during severe hypobaric hypoxia decreased serum VEGF level. Serum VEGF concentration is determined by its production as well as the release, removal, and binding of circulating VEGF [14]. Our findings of an insignificant association between VEGF mRNA expression level and VEGF protein concentration raises the possibility that changes in serum VEGF concentration after hypoxic exposure may be regulated by factors other than transcriptional upregulation at a cellular level.

To investigate whether the upregulation of serum VEGF mRNA expression was associated with elevated hepatic VEGF mRNA production, we determined VEGF mRNA levels in liver tissue homogenates obtained from hypoxic mice. We did not observe any statistically significant increase in hepatic VEGF mRNA compared to the control group, suggesting that the liver is unlikely to be the source of elevated serum VEGF mRNA. However, as only a small number of animals were used in the study and the exposure duration was short, the possibility of an association between hepatic VEGF mRNA production and serum mRNA level cannot be completely discounted.

In conclusion, the results of the present study indicate that hypobaric hypoxia upregulates serum mRNA levels of HIF-1α and VEGF in mice. The liver seems unlikely to be the source of elevated serum VEGF mRNA. Our findings of an insignificant association between level of serum VEGF mRNA and protein concentration indicate that changes in serum VEGF concentration after hypoxic exposure are possibly regulated by factors other than transcriptional upregulation. Further investigations to confirm or disprove our preliminary results are required.

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Disclosure of conflict of interest

None.

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