Original Article
Effect of intermittent hypoxia on osteoprotegerin expression in rat liver

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Abstract: Hypoxia is one of the most frequently encountered stress factors in both health and disease. Intermittent hypoxia (IH) is the most common pattern of hypoxic exposure. Osteoprotegerin (OPG) is produced by various cell types, including hepatocytes. Alterations in serum and tissue levels of OPG have been linked to many types of diseases, and clinical significance of OPG expression status has recently been clarified. However, the effect of IH on the expression of OPG in the liver remains unknown. The aim of this study was to investigate the effect of IH on hepatic OPG expression. Male Sprague-Dawley rats were placed in a hypobaric chamber (282 mm Hg, 30 min/d, 14 d). Hepatic OPG mRNA and protein levels were determined using quantitative real-time RT-PCR and immunohistochemical staining, respectively. A significant decline in hepatic OPG expression at both mRNA and protein levels was reported in the IH model. In hypoxic rats, IH significantly decreased the OPG mRNA expression, when compared with the normoxic control (P=0.001). Immunohistochemistry revealed that the normoxic livers exhibited a moderate to strong, homogeneous cytoplasmic staining of hepatocytes. In contrast, all the hypoxic livers showed a significantly reduced OPG staining intensity, when compared with the normoxic livers. In 9 of the 12 (75.0%) hypoxic rats, hepatic OPG immunoreactivity was undetectable. Our preliminary data suggest that IH exposure downregulates hepatic OPG at both the transcriptional and translational levels. Further investigations are necessary to clarify the mechanism by which hypoxia would induce OPG downregulation.

Keywords: Intermittent hypoxia, osteoprotegerin, rat, liver

Introduction
The aerospace environment has negative effects on bodily function, resulting in malfunction of vital organs. Flight surgeons continually seek to determine and understand pilots’ physiological responses to hypoxia and the pathophysiological mechanisms involved. Advanced aircraft design has made it increasingly possible to fly at higher altitudes. However, the possibility of experiencing hypoxia still exists, not only in pilots and cabin crews but also in patients who are being transported by military or civilian airlines [1]. Because symptoms of hypoxia can vary with each individual, the ability to recognize and address hypoxia can be greatly improved by experiencing and observing its effects. It is critically important that pilots, cabin crews, and medical professionals should be very familiar with hypoxia and the factors that affect its presentation.

Hypoxia is one of the most frequently encountered stress factors in both health and disease, and it is often implied as the common cause of tissue and cell injury. However, recent studies have indicated that the effects of hypoxia on the body can vary depending on the duration, severity, and frequency of hypoxic exposure [2]. Individuals may be exposed to acute, chronic, or intermittent hypoxia (IH) during their life. The effects of acute and chronic hypoxia have been assessed over many decades. However, studies on IH, the most common pattern of hypoxic exposure, have just begun in recent years. IH is defined as repeated episodes of hypoxia interspersed with normoxic breathing [2]. IH may be observed in both physiological and pathophysico-
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Osteoprotegerin (OPG) has been recently identified as a member of the tumor necrosis factor (TNF) receptor family, whose best characterized activity is the inhibition of receptor activator of nuclear factor-κB ligand-stimulated formation of osteoclasts [3-5]. OPG can also stimulate cell survival by acting as a receptor for TNF-related apoptosis-inducing ligand (TRAIL) [6]. There is growing evidence that OPG plays key roles in regulating numerous physiological and pathophysiological processes. It is widely accepted that OPG plays a central role in the regulation of bone turnover and a number of studies have assessed the role of OPG in bone-related diseases including osteoporosis, Paget’s disease, and arthritis [7-10]. Moreover, OPG appears to have a vascular role, and in particular, it appears to prevent processes involved in atherosclerosis [11, 12]. With respect to tumorigenesis, the role of OPG appears to be more complex. The ability of OPG to inhibit the development of cancer-induced bone diseases suggests that OPG is effective at inhibiting this process [13]; however, paradoxically, evidence that OPG can inhibit TRAIL-induced apoptosis of cancer cells suggests that OPG is pro-tumorigenic [14]. Although the role of OPG in a number of diseases is beginning to emerge, there is a need to further elucidate whether OPG is protective or detrimental, and potential mechanisms by which OPG may act.

It has been shown that exposure to hypoxia affects the production and release of OPG, but the effect of IH on OPG expression remains unknown. Furthermore, alteration in the hepatic expression of OPG following exposure to IH has not yet been investigated. The aim of this study was to examine the expression of OPG mRNA and protein in the livers of rats exposed to 14 d of IH and to investigate whether exposure to IH can lead to significant changes in the hepatic OPG expression when compared with the normoxic control.

Materials and methods

Experimental animals

Sixteen male Sprague-Dawley rats weighing between 200 and 230 g were used. The animals were randomly divided into IH and normoxic control groups. To obtain IH, 12 rats were placed in an altitude chamber at a barometric pressure of 282 mm Hg, corresponding to an altitude of 7,620 m (approximately 25,000 ft; equal to 7% oxygen at sea level). The temperature and moisture of the chamber were maintained at 20-24°C and 45%, respectively, with 12-h light and dark cycles. The rats of the IH group (n=12) were kept at this barometric pressure for 30 min/d for 14 d. The rats of the control group (n=4) stayed in the same environment as those of the IH group with access to food and water ad libitum, with the exception of breathing normal room air. The chamber was opened daily to clean the cages and replenish food and water. At the end of exposure to IH, both groups of animals were anesthetized by carbon dioxide. For tissue collection, the rats were laparotomized via a midline incision. A portion of each liver was fixed in 10% neutral buffered formalin for immunohistochemical staining. The remaining tissue was partitioned and immediately stored under frozen liquid nitrogen at -80°C until reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed. The care of animals and euthanasia procedures were performed in accordance with the guidelines established by the National Institute of Health. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Republic of Korea Air Force Aerospace Medical Center (Protocol No.: ASMC-14-IACUC-003).

Quantitative real-time reverse transcriptase-polymerase chain reaction analysis

Quantitative real-time RT-PCR was performed on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the C1000 Thermal Cycler (Bio-Rad Laboratories). Total serum RNA was isolated using the NucleoSpin RNA II extraction kit (Macherey-Nagel, Düren, Germany), according to the manufacturer’s instructions. cDNA synthesis was performed with the ReverTra Ace-α-reverse transcriptase kit (Toyobo, Osaka, Japan), according to the manufacturer’s instructions. The amount of standard cDNA was determined photometrically. The reverse-transcribed cDNA was used for the real-time RT-PCR reaction using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories). The following primer
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The sequence of OPG used: forward 5'-GGCAAGTTGACGGTGTAGC-3'; reverse 5'-GGAACAAGAAACACTGGACTC-3'. The following primer sequence of GAPDH was used: forward 5'-CATAGACAAGATGGTGAAGGT-3'; reverse 5'-AGTTGAGGTCAATTGAAGGG-3'. The PCR reactions for OPG and GAPDH were 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, ramping from 65°C to 95°C, was performed following each RT-PCR to test for the presence of primer dimers. When primer dimer formation was detected, the PCR was run again using a separate aliquot of cDNA. Each measurement was repeated three times, and the values were used to calculate the ratio of OPG/GAPDH, with a value of 1.0 used as the control (calibrator).

Immunohistochemistry

Formalin-fixed liver tissue was dehydrated in a graded series of ethanol and embedded in paraffin. Paraffin blocks were sectioned at 4 μm on a standard rotary microtome, and the slices were transferred from a water bath onto cleaned slides. OPG protein expression was assessed by immunohistochemical staining using the Bond Polymer Intense Detection System (Vision Bio-Systems, Mount Waverley, Victoria, Australia), following the manufacturer's instructions. To summarize, 4-μm sections of formalin-fixed, paraffin-embedded tissue were deparaffinized with Bond Dewax Solution (Vision Bio-Systems), and an antigen retrieval procedure was performed using Bond ER Solution (Vision BioSystems) for 30 min at 100°C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 min. The sections were incubated for 15 min at ambient temperature with a rabbit polyclonal anti-OPG antibody (1:100, Abcam, Cambridge, MA, USA). The biotin-free polymeric horseradish peroxidase-linker antibody conjugate system was used in the Bond-maX automatic slide stainer (Vision BioSystems), and visualization was performed by using a 3.3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6] and 0.006% H2O2). The sections were then counterstained with hematoxylin. Slides were subsequently dehydrated following a standard procedure and sealed with coverslips. In order to minimize interassay variation, positive and negative control samples were included in each run. The positive control sample was normal liver tissue. The negative control was prepared by substituting non-immune serum for the primary antibody; no detectable staining was evident.

The immunohistochemical staining was analyzed by an experienced pathologist. The OPG
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staining intensity of the hepatocytes in each sample was graded as negative, weak, moderate or strong, as described previously [15]. There was no heterogeneity of staining within individual slides; estimation of the proportion of staining was not required. Disagreements between the two pathologists were resolved by consensus.

Statistical analysis

Measurements are presented as mean ± standard error. Statistical significance of the difference in OPG mRNA levels between control and IH groups was determined by the Mann-Whitney U-test. The Fisher’s exact test was performed to compare OPG protein expression between control and IH groups. The relationship between the levels of OPG protein and mRNA expression was examined using the Spearman’s rank correlation coefficient. Statistical analyses were performed using the PASW Statistics for Windows (version 18.0; IBM SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a P value of less than 0.05.

Results

OPG mRNA expression in the livers of rats exposed to IH

We performed real-time RT-PCR to measure the expression of OPG mRNA transcripts in 4 fresh normoxic livers and 12 fresh hypoxic livers. Compared with the normoxic livers, the hypoxic livers showed reduced expression levels of OPG mRNA. Eleven (91.6%) of the 12 rats exposed to IH showed lower levels of OPG mRNA expression than the normoxic rats (Figure 1A). The mean value of normalized OPG/GAPDH mRNA expression ratio in the IH group (0.411 ± 0.088; range, 0.128-1.034) was significantly lower than that in the control group (2.868 ± 0.808; range, 1.000-4.576) by 0.15-fold (P=0.001).

OPG immunoreactivity in the livers of rats exposed to IH

Representative photomicrographs of OPG staining intensity are shown in Figure 1B. Immunohistochemical staining revealed that the normoxic livers exhibited a moderate to strong, homogeneous cytoplasmic staining of hepatocytes. OPG was expressed in the cytoplasm of hepatocytes, as well as in other cell types, including bile duct epithelial cells and activated Kupffer cells, and in inflammatory cells including plasma cells and lymphocytes. In contrast, all the hypoxic livers showed a significant reduction in staining intensity of OPG-positive hepatocytes, when compared with the normoxic livers. In 9 of the 12 (75.0%) hypoxic rats, hepatic OPG immunoreactivity was undetectable (negative; Table 1). Of the remaining 3 hypoxic rats, 2 (16.7%) rats displayed weak staining intensity, and 1 (8.3%) rat exhibited moderate staining intensity. Two (50.0%) of the 4 control rats had strong OPG immunoreactivity, and the remaining 2 (50.0%) had moderate reactivity. Correlation between mRNA expression and immunohistochemical staining intensity was statistically significant (P<0.001; rank correlation coefficient, 0.860).

Discussion

Simonet and colleagues [4] observed OPG mRNA expression in a number of murine tissues, including liver, lung, heart, and kidneys. Yasuda and colleagues [5] also showed that OPG is highly expressed in human liver, thyroid, kidney, colon, and spinal cord. Even though OPG is known to have an important role in osteoclastogenesis, a variety of cellular functions of OPG have been noted in different types of human tissues and cells. Vidal and colleagues [16] reported that OPG is constitutively produced by human colonic epithelium, and OPG may represent an important mucosal immunoregulatory factor.

Some previous studies have documented OPG expression in normal and diseased livers. Moschen and colleagues [17] demonstrated a moderate, uniform cytoplasmic OPG immunoreactivity in the hepatocytes of normal human liver. Guanabens and colleagues [18] showed

<table>
<thead>
<tr>
<th>OPG staining intensity</th>
<th>Number of samples (%)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Strong</td>
<td>Control group</td>
<td>IH group</td>
</tr>
<tr>
<td>Strong</td>
<td>2 (50.0)</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>2 (50.0)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>9 (75.0)</td>
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*Statistically significant.
that the serum OPG level is significantly altered in patients with chronic liver disease. The role of OPG has been studied in several types of liver disease, but the association between exposure to hypoxia and hepatic OPG expression has not yet been investigated.

We observed that exposure to IH resulted in a significant decrease in OPG mRNA and protein expression. Our results are in accordance with previous data reported by Shirakura and colleagues [19] who demonstrated that hypoxia significantly downregulated the expression of OPG mRNA and protein in mouse chondrocytes and human oral squamous cell carcinoma cells. There is no available data on the mechanism by which hypoxia would induce OPG downregulation. Based on the finding that activated p53 pathway downregulates OPG mRNA and protein expression in vascular endothelial cells [20], we speculated that hypoxia might be involved in the downregulation of OPG expression in the liver of rats by activating p53. The p53 protein has been reported to be stimulated and to accumulate in different cell lines under hypoxia [21]. This accumulation may increase with the duration of hypoxia incubation as well as the decrease in the oxygen level. Zhao and colleagues [22] observed that hypoxic exposure mimicking an altitude of 5,000 m for 8 h significantly increased TP53 mRNA and p53 protein expression levels in the liver of rats, and at an altitude of 7,000 m p53 expression was further increased. Moreover, when the 7,000 m challenge was extended to 24 h, the p53 mRNA level was maintained at a significantly high level. Further investigations are warranted to clarify the relationship between p53 and OPG expression in hypoxic conditions.

In contrast, contradictory data have been reported. Gao and colleagues [23] demonstrated that OPG expression was increased under hypoxic conditions in metastatic hepatocellular carcinoma cells. Meanwhile, Mandelin and colleagues [24] found that the pro-inflammatory cytokine TNF-α caused a marked increase in OPG mRNA levels in human endothelial cells, whereas hypoxia did not stimulate the endothelial cells to produce OPG mRNA. These findings raise the possibility that hypoxia was less likely to modulate the TNF-α-induced OPG production and that OPG expression in human endothelial cells was not dependent on the oxygen level.

In conclusion, our preliminary data demonstrated that IH exposure downregulated hepatic OPG at both the transcriptional and translational levels. Further investigations are necessary to clarify the mechanism by which hypoxia would induce downregulation of hepatic OPG expression.

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Disclosure of conflict of interest

None.

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