Hyperbaric oxygen-stimulated proliferation and growth of osteoblasts may be mediated through the FGF-2/MEK/ERK 1/2/NF-κB and PKC/JNK pathways

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ABSTRACT

We investigated whether the hyperbaric oxygen (O2) could promote the proliferation of growth-arrested osteoblasts in vitro and the mechanisms involved in this process. Osteoblasts were exposed to different combinations of saturation and pressure of O2 and evaluated at 3 and 7 days. Control cells were cultured under ambient O2 and normal pressure [1 atmosphere (ATA)]; high-pressure group cells were treated with high pressure (2.5 ATA) twice daily; high-O2 group cells were treated with a high concentration O2 (50% O2) twice daily; and high pressure plus high-O2 group cells were treated with high pressure (2.5 ATA) and a high concentration O2 (60% O2) twice daily. Hyperbaric O2 significantly promoted osteoblast proliferation and cell cycle progression after 3 days of treatment. Hyperbaric O2 treatment stimulated significantly increased mRNA expression of fibroblast growth factor (FGF)-2 as well as protein expression levels of Akt, p70S6K, phosphorylated ERK, nuclear factor (NF)-κB, protein kinase C (PKC)α, and phosphorylated c-Jun N-terminal kinase (JNK). Our findings indicate that high pressure and high O2 saturation stimulates growth-arrested osteoblasts to proliferate. These findings suggest that the proliferative effects of hyperbaric O2 on osteoblasts may contribute to the recruitment of osteoblasts at the fracture site. The FGF-2/MEK/ERK 1/2/Akt/p70S6K/NF-κB and PKC/JNK pathways may be involved in mediating this process.

KEYWORDS: hyperbaric O2; osteoblast; FGF-2/MEK/ERK 1/2/NF-κB pathway; PKC/JNK pathway; cell cycle

INTRODUCTION

Nonunion or delayed union, a complication occurring in up to 10% of fractures, not only presents a challenge for physicians with regard to clinical management but may result in prolonged disability and economic loss for the patient [1]. In the past, orthopedic surgeons have treated nonunion and delayed union with a combination of internal fixation and surgical decortication and autogenous bone grafting [1]. These methods are not always successful, can be uncomfortable for patients, and are associated with the risk of wound infection. Interestingly, electromagnetic stimulation has proven to be an effective adjunct to conventional therapy when used in the management of nonunion of long bone fractures [1,2]. Other techniques, including hyperbaric O2 therapy (HBOT), have also been examined as more efficacious treatment alternatives [1]. However, additional quality clinical trials are needed to irrefutably establish the effectiveness of HBOT for the management of delayed union or nonunion of bony fractures [3].

Localized hypoxia is common in the microenvironment of a bone or soft tissue injury site [4] and has been reported to be a key limiting factor in the healing of dermal wounds [5]. The greatest extent of hypoxia is found in the central area of the wound, where the O2 pressure ranges from 0 to 10% of arterial O2 pressure [5]. The degree of hypoxia decreases toward the uninjured tissue at the edge of the wound, where the O2 pressure may be 60% of arterial O2 pressure [5]. Although hypoxia at a wound site may impede the vital processes of damage repair, including angiogenesis, inflammatory cell recruitment, extracellular matrix formation, and the activation of mesenchymal osteoblast precursors [5,6], the gradient O2 gradient is an important contributor to wound healing and is accentuated by HBOT. Indeed, the treatment of experimental dermal wounds with HBOT
significantly reduces post-burn edema, stimulates neoangiogenesis, preserves regenerative active follicles, and reduces the time required for epithelial regeneration [7].

HBOT has been reported to be beneficial for the treatment of delayed union and nonunion of fractures [8]. For appropriate patients, hyperbaric O2 treatment can significantly reduce the length of hospital stay, amputation rate, and cost of care [8]. Studies utilizing animal models have also found HBOT to be beneficial in some conditions for bone healing. It has been demonstrated that HBOT promotes osteogenesis in osteoradionecrosis by increasing osteoblast activity and neoangiogenesis [9,10]. HBOT has also been reported to increase the accumulation of minerals needed for osteogenesis, such as calcium, magnesium, and phosphorus [11,12]. Furthermore, hyperbaric O2 may help accelerate bone repair by encouraging vessel ingrowth [13], migration of connective tissue from surrounding soft tissue [14], increasing bone mineral density [15,16], and enhancing osteogenesis in the section of fracture [17]. Other researchers have found that hyperbaric O2 treatment enhances healing in ungrafted, critical-size calvarial defects, may increase the rate of residual graft resorption in autogenous bone-grafted defects [18], augment the healing process in distraction osteogenesis [19], and enhance fibroblastic, angioblastic, osteoblastic, and osteoclastic activities in rat vascular deprivation-induced necrotic femoral heads [20].

Multiple signal transduction pathways have been shown to be involved in the regulation of osteoblast-specific transcription [21]. These signal pathways mostly correlate with environmental stimuli, including osteogenic growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF)-2, extracellular matrix, mechanical loading, and hormones such as parathyroid hormone [21]. Protein kinase A (PKA) and protein kinase C (PKC) are also two well-defined mediators of osteoblast proliferation. These kinases activate the formation of 1,4,5-inositol trisphosphate, which stimulates an increase in intracellular free Ca2+ and related signaling events. Specifically, the activation of PKA and PKC can facilitate regulation of transcription factors such as cAMP response element-binding proteins, AP-1 family members, and Runx2 [22]. PKC may be an intermediate in the activation of c-Jun N-terminal kinase (JNK) during hypoxia–reoxygenation [23].

Endogenous growth factors such as insulin-like growth factors, transforming growth factor (TGF)-β, and BMPs produced by osteoblasts may play an important role in osteogenesis and differentiation during the early stages of bone injury [24]. In vivo studies have demonstrated that the application of these growth factors may systemically help to increase bone formation, promote fracture healing, and induce bone growth around the fracture site [24].

A retrospective study of calvarial critical-sized defects in rabbits revealed that HBOT resulted in increased vascular endothelial growth factor (VEGF) expression 2 weeks after the termination of treatment [25]. In another study, it was found that hyperbaric O2 treatment of fibroblasts led to an increased production of the growth factors VEGF and FGF [26]. Microarray analysis has revealed that osteoblasts exposed to hyperbaric O2 conditions in vitro differentially express a variety of gene families [27]. It is not understood how hyperbaric O2 treatment promotes osteoblastic and angiogenic growth, but based on these and other observations, enhanced production of growth factors has been proposed as a possible mechanism for the observed effects [28].

Given the aforementioned findings, we hypothesized that osteoblasts possess the ability to respond to hypoxia directly, leading to changes in cell-signaling pathways involved in cell proliferation and growth factor production. To test this hypothesis we exposed mouse osteoblasts to different O2 and pressure conditions and examined proliferation, cell cycle profiles, levels of expression of several growth factors, and their intermediates in downstream signaling pathways.

**MATERIALS AND METHODS**

**Cell culture and biological regimens**

The mouse osteoblast-like immortalized cell line MC3T3-E1 (American Type Culture Collection, Manassas, Va, USA) was maintained in α-minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (0.0002 g/L) (Sigma, St. Louis, Mo, USA). Early passage (<3) cells were utilized for experimentation. Osteoblasts were seeded at a density of 1 × 10^4 cells/well in 6-well plates, with a final volume of 100 µL in all wells. All cells were maintained at 21% O2 and 5% CO2 in humidified incubators at 37°C before exposure to various O2 tension and pressure conditions. Cell growth medium was changed every 2 days. For cell growth arrest, cells were cultured in serum-free medium for 48 hr.

**Exposure to various O2 tensions and pressure conditions**

For O2 tension and pressure experiments, osteoblasts were transferred to a hyperbaric O2 chamber (Sigma II, Perry Baromedical, Riviera Beach, Fl, USA). The culture plates were sealed in a sterile hood and flushed with different concentrations of O2 and various levels of atmospheric pressure. Temperature was controlled within 3°C of 37°C.

Conditions combining different O2 concentrations and levels of pressure were used to investigate the effects of O2 on osteoblast proliferation. Experiments incorporated four
Hyperbaric oxygen-stimulated proliferation and growth of osteoblasts

FIGURE 1 Morphology of cells in different experimental groups after 7 days of hyperbaric oxygen treatment. The cell density of treated groups significantly increased compared with the control group. C, control group (untreated/normal pressure cells); P, cells exposed to high pressure twice daily; O, cells exposed to a high concentration of oxygen twice daily; P + O, cells exposed to both high pressure and high oxygen concentration twice daily.

FIGURE 2 Net cell proliferation rates [Δ optical density (ΔOD)] of osteoblasts after 3 and 7 days of treatment. C, control group (untreated/normal pressure cells); P, cells exposed to high pressure twice daily; O, cells exposed to a high concentration of oxygen twice daily; P + O, cells exposed to both high pressure and high oxygen concentration twice daily. Significant differences between groups are as indicated.

FIGURE 3 Cell cycle profiles of osteoblasts after 3 and 7 days of treatment as determined by flow cytometric analysis. C, control group (untreated/normal pressure cells); P, cells exposed to high pressure twice daily; O, cells exposed to a high concentration of oxygen twice daily; P + O, cells exposed to both high pressure and high oxygen concentration twice daily. Significant differences between groups are as indicated.
groups: (1) control group (C) cells were cultured under ambient O₂ (21% O₂) and normal pressure [1 atmosphere (ATA)]; (2) high-pressure group (P) cells were treated with high pressure (2.5 ATA); (3) high-O₂ group (O) cells were treated with a high concentration of O₂ (50% O₂); (4) high pressure plus high-O₂ (P + O) group cells were treated with high pressure (2.5 ATA) and high concentration O₂ (50% O₂). Each exposure to 2.5 ATA and/or 50% O₂ was 15 and 5 min break under ambient O₂ and 1 ATA. The steps outlined above were repeated four times (for a total of total 80 min) twice daily.

Evaluation of cell proliferation and cell cycle progression

Cell proliferation was examined using XTT labeling reagents (Cell Proliferation Kit II, Roche Molecular Biochemicals, Indianapolis, IN, USA). Cells were treated for 3 or 7 days, and then the XTT reagent was added to each cell culture well. The cells were incubated with the reagent for 4 hr, and then cell proliferation was determined by assessing differences from day 0 in absorbance at 490 nm.

RNA extraction and RT-PCR

Total RNA was isolated from control and treated cells following 0, 3, and 7 days of culture using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). cDNAs were primed with oligo(dT)12-18 and extended using reverse transcriptase (RT) (Clontech, Mountain View, CA, USA). cDNAs were amplified by polymerase chain reaction (PCR) using the following primer pairs: BMP-2 forward, 5’-AAGAAGCCATCGAGGAACTTTCAG-3’; BMP-2 reverse, 5’-CCTGAGAC-CAGCTGTGTTCATCTT-3’; VEGF forward, 5´-ACCCTGGCTTTACTGCTGTACCTC-3´; VEGF
Hyperbaric oxygen-stimulated proliferation and growth of osteoblasts

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reverse, 5’-TCACCGCCTTTGCTTGTCACA-3’; FGF-2 forward, 5’-AAGCGGCTCTACTGCAA-GAA-3’; and FGF-2 reverse, 5’-CCGTTTTGGGATC-CGAGTTTA-3’.

PCR products were separated on 2% agarose gels in tris-acetate-EDTA (TAE) buffer and after the resultant bands were visualized by staining the gels with ethidium bromide. Band-intensity signals were quantified using an Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA) and normalized against β-actin.

Protein extraction and western blot analysis

Total cellular proteins were extracted from control and experimental cells using lysis buffer. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Antibodies against Akt, Ser473-phosphorylated Akt, p70S6K, phosphorylated p70S6K (Thr421/Ser424), ERK1/2, phosphorylated ERK1/2, JNK, phosphorylated JNK, P-38, phosphorylated P-38, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against nuclear factor (NF)-κB, PKC, phosphorylated-PKC, and cyclin-D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL, USA).

Cell proteins were fractionated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and then transferred onto nitrocellulose membranes, blocked, and probed with various primary antibodies (1:1000) overnight at 4°C. Following incubation, membranes were washed and incubated with the

FIGURE 5 FGF-2 and BMP-4 mRNA expression levels in osteoblasts after 3 and 7 days of treatment. Representative blots are shown. C, control group (untreated/normal pressure cells); P, cells exposed to high pressure twice daily; O, cells exposed to a high concentration of oxygen twice daily; P + O, cells exposed to both high pressure and high oxygen concentration twice daily. Significant differences between groups are as indicated.

1.4
1.2
1.0
0.8
0.6
0.4
0.2

CPO P  + O CPO P  + O

FGF/β-actin FGF/β-actin

p < 0.01
p < 0.01

3 days 7 days

p < 0.01
p < 0.05
p < 0.01

3 days 7 days
appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000) for 1 hr. Blots were washed again and protein bands were visualized using enhanced chemiluminescence (Pierce Biotechnology). Specific bands were quantified and normalized against β-actin.

**Statistical analysis**

Statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. Differences between groups were evaluated using one-way analysis of variance followed by Tukey’s post hoc tests. Differences were considered statistically significant when p < 0.05.

**RESULTS**

**Osteoblast proliferation**

After treatment for 7 days, the morphology of cells in the control group (C), pressure group (P), high-O₂ group (O), and pressure plus high-O₂ group (P + O) was observed. It has been observed that the cell density of treated groups significantly increased (Figure 1).

The net proliferation rate (Δ optical density) was determined on days 3 and 7 of treatment. After treatment
for 3 days, the proliferation rates in all treatment groups were significantly higher than that in the control group (Figure 2, all $p < 0.05$). Among the three treatment groups, the P group had the highest proliferation rate, followed by the P + O group, and the O group. However, there were no significant differences among these groups. After treatment for 7 days, the proliferation rates of all three treatment groups were still significantly higher than that of the control group, with no differences between the treatment groups (Figure 2).

**Cell cycle progression**

The cell cycle profiles for each of the four groups as determined by flow cytometric analysis are summarized in Figure 3. Three days after treatment, significantly more cells in the O and the P + O groups were in the S and G2/M phases relative to those in the control group (both $p < 0.05$).

Cyclin-D1 is an important regulator protein in the early G1 phase of the cell cycle [29]; therefore, we examined protein expression levels in growth-arrested osteoblasts stimulated with hyperbaric O$_2$ treatment for 3 and 7 days. Compared to the control and O groups, the expression level of cyclin D1 was significantly increased in the P and P + O groups following stimulation for 3 days ($p < 0.01$, Figure 4). After treatment for 7 days, the expression levels of cyclin D1 in the three treatment groups were significantly higher than that in the control group (Figure 4).

**Growth factor expression levels**

To investigate whether hyperbaric O$_2$ treatment could stimulate the expression of growth factors, we examined the mRNA expression levels of BMP-2, BMP-4, VEGF, and FGF-2 using RT-PCR. There were no differences in the transcriptional expression levels of BMP-2 and VEGF between the control group and the treatment groups (data not shown). BMP-4 was undetectable at the 3-day time point in any treatment group (Figure 5). However, at day 7, BMP-4 was detected in the control group. Although BMP-4 expression was somewhat suppressed in the P and P + O groups and enhanced in the O group, there were no significant differences between groups at either time point. On day 3 of treatment, the level of expression of FGF-2 in the O group and the P + O groups was significantly higher than that in the control group ($p < 0.05$, Figure 5). On day 7 of treatment, FGF-2 expression levels were significantly higher in all three treatment groups compared with that in the control group ($p < 0.05$).
Expression of factors downstream from FGF-2

To investigate the possible activation by FGF-2 of downstream signaling pathways, we examined protein expression levels of Akt, ERK, NF-κB, JNK, and PKCα. Akt expression levels were significantly higher in all treatment groups compared with that in the control group at both days 3 and 7 of treatment (Figure 6A, all \( p < 0.05 \)). After 3 days of treatment, \( p70S6K \) expression was significantly higher in the P + O group compared with that in the control group (\( p < 0.01 \)). Although at 7 days, \( p70S6K \) expressions levels were significantly higher in the P + O and the O groups compared with that in the control group (Figure 6B, both \( p < 0.05 \)). On day 3 of treatment, expression of phosphorylated ERK1/2 was significantly decreased in the P + O group compared with that in the control group (Figure 7A, \( p < 0.05 \)). However, on day 7, ERK1/2 expression levels were significantly higher in all treatment groups versus the control group (Figure 7A, all \( p < 0.05 \)). Expression of NF-κB, a target protein of Akt and mitogen-activated protein kinase (MAPK), was also significantly elevated in all treatment groups on both days 3 and 7 (Figure 7B, all \( p < 0.05 \)). On days 3 and 7 of treatment, all treatment groups exhibited higher levels of expression of both phosphorylated PKCα (Figure 8A) and phosphorylated JNK (Figure 8B) than the control group (all \( p < 0.05 \)).

DISCUSSION

Existing treatment modalities for fracture reconstruction produce excellent results in most cases; however,
delayed union and nonunion of fractures remains a major complication of fracture injuries. In some cases, factors leading to delayed union and nonunion can be identified, whereas in other cases, these factors remain unclear. It is thought that the proliferation and differentiation of osteoblasts plays a key role in the healing of fractures [30]. However, other unknown factors can lead to delayed or terminated bone healing.

Several studies have concluded that HBOT has no effect on osteoblast proliferation in vitro and may even result in apoptosis [31]. In a study by Wong et al., 100% O₂ at 2 ATA inhibited growth of primary osteoblasts and resulted in a significant increase in apoptosis [27]. In this study, the tension of O₂ was modified from 100 to 50%, and hyperbaric O₂ treatment was found to have a positive effect on osteoblast proliferation. The differences between the present results and those reported by Wong et al. may stem from the use of different cells (primary culture osteoblasts vs. an osteoblast cell line) and also the possible generation of excessive free radicals in a 100% O₂ environment. The O₂ tension in culture medium treated with 2.5 ATA and 50% O₂ is more comparable to the O₂ tension in muscle (about 300–400 mmHg) during clinical HBOT. Pure O₂ (100%) combined with 2.5 ATA would elevate the O₂ tension to a very high level (about 600–700 mmHg) in the culture medium, potentially resulting in cell damage [32]. It will be important to confirm the present results by applying the current treatment protocols to cultured primary osteoblasts and to other osteoblast cell lines.

Consistent with our observation that HBOT promotes osteoblast proliferation, results from our cell cycle profile experiment indicate that after 3 days of HBOT, growth-arrested osteoblasts are induced to enter the S and G2/M phases of the cell cycle. Because maintenance of bone homeostasis is a dynamic process involving both bone formation and bone resorption through the activity of osteoclasts, it is worth noting that other investigators have reported that hypoxia (2% O₂) stimulates osteoclast formation and activity [33]. Other investigators reported that 40% O₂ prolonged osteoclast precursor cell survival [34]. Thus, future investigations should consider the broader effects of O₂ tension on bone homeostasis in addition to its effects on particular cell types of factors influencing healing.

Multiple growth factors are involved in the process of osteogenesis [24,35]. In this study, we looked at the expression levels of BMP-2, BMP-4, VEGF, and FGF-2. Only the level of expression of FGF-2 was found to be significantly increased in response to HBOT. It is well known that HBOT induces FGF-2 expression in soft tissue healing, but no comparable
study has been performed with regard to bone healing. FGF-2 is an important regulator of bone differentiation and growth in vivo. In vitro, FGF-2 has been shown to stimulate osteoblast proliferation [36] and transforming growth factor-beta production [37]. The expression of FGF-2 contributes not only to soft tissue healing but also to angiogenesis [38] by further inducing the expression of VEGF in osteoblasts [39]. The cooperative interaction between vascular endothelial cells and osteoblasts as has been illustrated in previous studies demonstrates that several factors produced by endothelial cells can affect osteoblast function and differentiation [40,41].

The process of osteoblast proliferation can be regulated through multiple signal transduction pathways.

The results from our study suggest that hyperbaric O₂ treatment promotes the proliferation of osteoblasts through at least two signaling pathways. The FGF-2/MEK/ERK (MAPK) pathway may be the principal pathway involved. Evidence suggests that signaling through the MAPK pathway is essential for the early stage of osteoblast differentiation [42,43]. We examined the expression levels of several intermediates in the downstream signaling pathway. The observed significantly increased levels of phosphorylated ERK, P-38, Akt/p70S6K, and NF-κB strongly support the involvement of this pathway. The levels of phosphorylated PKC and phosphorylated JNK were also elevated, suggesting that JNK might be the major downstream kinase of the PKC signal in hyperbaric O₂-induced proliferation of osteoblasts. The levels of phosphorylated PKC and phosphorylated JNK were elevated to a lesser degree than the levels of phosphorylated ERK, P-38, Akt/p70S6K and NF-κB, perhaps suggesting an accessory role for this pathway. Further studies, for instance those involving the use of RNA interference, are warranted to confirm the postulated involvement of these pathways in osteoblast proliferation.

In our study, the partial pressure of O₂ was the same, 380 mmHg, in the P and O groups (P group: 760 mmHg × 20% × 2.5 ATA = 380 mmHg; O group: 760 mmHg × 50% × 1 ATA = 380 mmHg). Under the same partial pressure of O₂, we can compare the effects of pressure and O₂. From our results, we are not able to conclude that either of these factors plays a more important role than the other factor. The effects of increased pressure (the P group) and increased O₂ (the O group) seem not to be synergistic with regard to the expression of some signal proteins. Furthermore, we are unable to determine the optimum length of treatment from the findings presented. The level of expression of some signal proteins decreased on day 7 of treatment relative to the level on day 3 of treatment, thus it is possible that treatment longer than 3 days may suppress the expression of some signaling factors.

Our results show that a combination of 50% O₂ exposure and 2.5 ATA elicits the proliferation of osteoblasts in vitro. Furthermore, our findings also suggest that this effect may be mediated through enhanced expression of FGF-2 and the subsequent activation of the FGF-2/MEK/ERK/Akt/p70S6K/NF-κB pathway. Activation of the PKA/PKC/JNK pathway would also appear to be important. Although a systematic review failed to locate any relevant clinical evidence to support or refute the effectiveness of HBOT for the management of delayed union or established nonunion of bone fractures [2], our study suggests that a critical level of O₂ tension and
pressure may be required to stimulate osteoblast proliferation. Clinically, our findings indicate that the hyperbaric O2 stimulation of osteoblast proliferation may contribute to the recruitment of osteoblasts at the fracture site. Further research is needed to further elucidate/confirm the underlying mechanism(s) of this proliferative response. Hyperbaric O2 may ultimately prove to be a useful treatment in the clinical setting for the promotion of fracture repair in patients where healing is delayed or otherwise complicated.

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