Journal of Biomedical Science (2006) 13:143–156
DOI 10.1007/s11373-005-9037-7

Hyperbaric oxygen induces VEGF expression through ERK, JNK and c-Jun/AP-1 activation in human umbilical vein endothelial cells

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Received 17 February 2005; accepted 15 September 2005
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Key words: AP-1, c-Jun, ERK, hyperbaric oxygen, JNK, VEGF

Summary

Hyperbaric oxygen (HBO) is increasingly used in a number of areas of medical practice, such as selected problem infections and wounds. The beneficial effects of HBO in treating ischemia-related wounds may be mediated by stimulating angiogenesis. We sought to investigate VEGF, the main angiogenic regulator, regulated by HBO in human umbilical vein endothelial cells (HUVECs). In this study, we found that VEGF was up regulated both at mRNA and protein levels in HUVECs treated with HBO dose- and time-dependently. Since there are several AP-1 sites in the VEGF promoter, and the c-Jun/AP-1 is activated through stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and extracellular signal regulated kinase (ERK), we further examined the c-Jun, JNK and ERK that might be involved in the VEGF induced by HBO. The VEGF mRNA induced by HBO was blocked by both PD98059 and SP600125, the ERK and JNK inhibitors respectively. HBO induced phospho-ERK and phospho-JNK expressions within 15 min. We further demonstrated that c-Jun phosphorylation was induced within 60 min of HBO treatment. HBO also induced the nuclear AP-1 binding ability within 30–60 min, but the AP-1 induction was blocked by treatment with either the ERK or JNK inhibitor. To verify that the VEGF expression induced by HBO is through the AP-1 trans-activation and VEGF promoter, both the VEGF promoter and AP-1 driving luciferase activity were found increased by the cells treated with HBO. The c-Jun mRNA, which is also driven by AP-1, was also induced by HBO, and the induction of c-Jun was blocked by ERK and JNK inhibitors. We suggest that VEGF induced by HBO is through c-Jun/AP-1 activation, and through simultaneous activation of ERK and JNK pathways.

Introduction

Hyperbaric oxygen (HBO) is increasingly used in a number of areas of medical practice and has been used primarily in the treatment of carbon monoxide poisoning, air embolism, and several conditions, such as selected problem infections and wounds [1–4]. HBO also attenuates microvascular dysfunction and reperfusion microvascular ischemia, as demonstrated in both experimental models and patients with acute myocardial infarction [5]. Since molecular oxygen is one of the critical nutrients and plays a central role in the reparative process of wound [6], the beneficial effects of HBO in treating

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ischemia-related wounds may be mediated by stimulating collagen synthesis [7], cell proliferation [8–11], and promoting angiogenesis [12–14]. However, the mechanism(s) accounting for HBO-induced vessel formation is still not well documented.

Angiogenesis is the growth of new vascular capillary from preexisting vessels. Angiogenesis is required for the maintenance of functional and structural integrity of the organism during postnatal life. Thus, it occurs during wound healing, in inflammation, and in situations of ischemia. In response to a local angiogenic stimulus, endothelial cells of preexisting capillaries or postcapillary venules become activated. Although some non-vascular endothelium-specific growth factors participate in vessel formation [15], vascular endothelium-specific growth factors, such as vascular endothelial growth factors (VEGF) and angiopoietins, etc., are the primary regulators [16–18]. Among them, VEGF is the most critical driver of angiogenesis. In our previous study, we investigated the biological effects of HBO on human umbilical vein endothelial cells (HUVECs) because endothelial cells are the workhorses in angiogenesis. We have examined the expression of VEGF and the most prominent coworkers for vessel formation, angiopoietin 1 (Ang1), Ang2 and their receptor, Tie2, in the first hour of HBO treatment [19]. Our data indicated that HBO specifically stimulated the expression of Ang2. However, we did not find any change in VEGF expression during the first hour of HBO treatment.

In this study, we extended the time interval of HBO treatment on HUVECs to demonstrate that the VEGF is up regulated at both transcription and translation steps. VEGF promoter has been reported to have AP-1 transcription elements [20]. The c-Jun/AP-1 is activated through the mitogen-activated protein (MAP) kinases, such as stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and extracellular signal regulated kinase (ERK) [21–25]. We demonstrated that the expression of VEGF induced by HBO was simultaneous through the ERK and JNK pathways, and c-Jun/AP-1 activation.

Materials and methods

Chemicals and reagents

VEGF mouse monoclonal antibody was purchased from R&D System, Inc. (Minneapolis, MN, USA). Phospho-ERK, ERK, phospho-JNK and JNK rabbit polyclonal antibodies were bought from Cell Signaling (Beverly, MA, USA). α-tubulin mouse monoclonal antibody was gotten from Zymed Laboratories, Inc. (South San Francisco, CA, USA). The horseradish peroxidase-conjugated polyclonal anti-rabbit and anti-mouse antibodies were procured from Calbiochem (San Diego, CA, USA). Genistein, SB 203580, SP600125, SU1498 and U0126 were obtained from Calbiochem (La Jolla, CA, USA). PD98059 and wortmannin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase and Taq DNA polymerase were bought from Roche Applied Science (Indianapolis, IN, USA). M-199 medium, Penicillin–Streptomycin–Glutamine (100×), fetal bovine serum, and SuperScript™ II Reverse Transcriptase were gotten from Invitrogen Corporation (Carlsbad, CA, USA). α-^{32}P-dCTP, polyvinylidene difluoride membrane, Nylon membrane (Hybond-N+), the enhanced chemiluminescence (ECL) detection system, random prime labeling system (Rediprime), and hybridization buffer (Rapid-hyb buffer) were procured from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). N (omega)-nitro-L-arginine methyl ester (L-NAME), phorbol 12-myristate 13-acetate (PMA) and DMSO were obtained from Sigma-Aldrich Corporate (St. Louis, MO, USA). EGM was purchased from Clonetics (San Diego, Cam USA). The coomassie blue protein assay was gotten from Bio-Rad Laboratories, Inc. (Richmond, CA, USA). The oligo deoxy-nucleotides were synthesized by Mission Biotech (Taipei Taiwan). QIA quick gel extraction kit was obtained from Qiagen Inc. (Germantown, MD, USA). Tri-Reagent was bought from Molecular Research Center, Inc. (Cincinnati, OH, USA). Lipofectin Transfection Reagent was gotten from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase™ Reporter Assay System was obtained from Promega (Madison, WI, USA).

Primary human umbilical vein endothelial cells (HUVECs) culture, CL1-0 lung cancer cells culture and cells treatment

HUVECs were isolated from umbilical cord vein by collagenase treatment as previously described [26] and grown in EGM (Clonetics (CC-4133, Cambrex Bio Science Walkersville, Inc. Walkersville, MD, USA) supplemented with 12% fetal

bovine serum (Gibco, Invitrogen Corporation, Grand Island, NY, USA). After the cells fully grew, HUVECs were divided. Then the cells were seeded in the medium containing 2% fetal bovine serum overnight before treatment. The cells were incubated in the environment of 37 °C and 5% CO_2. HUVECs were used between passages 3 and 7. Lung cancer cells, CL1-0 (kindly gotten from Dr. Yi-Wen Chu), were cultured with RPMI medium plus 10% fetal bovine serum. For HBO treatment, cells were exposed to 2.5 or 1 ATA (atmosphere absolute) of oxygen (98% oxygen plus 2% CO_2) in a hyperbaric chamber for different time intervals at 37 °C. For the inhibition of signal pathways, cells were pretreated with inhibitors for 30 min, and then exposed to HBO without changing medium.

Northern blot analysis

Northern blot was used to detect mRNA expression. The VEGF, c-Jun and 28S probes for Northern blot analysis were synthesized by RT-PCR. The reverse transcription (RT) was performed as described in the manual of SuperScript™ II Reverse Transcriptase (Invitrogen Corporation, CA, USA). The primers used for amplification of VEGF (386 bp) were forward, 5′-cagattatgcggatcaaacct-3′, and reverse, 5′-acgttcgtttaactcaagct-3′; c-Jun (959 bp) forward, 5′-tgctgcgttagcatgagttg-3′, and reverse, 5′-gaccttctatgacgatgccc-3′; 28S (225 bp) forward, 5′-ccaagcgttggattgttcac-3′, and reverse, 5′-gattctgacttagaggcgttc-3′. The PCR product was purified with QIA quick gel extraction kit. About 20 ng of DNA fragment was labeled with α-^{32}P-dCTP by the random prime labeling system for Northern blot. The Northern blot was processed as described previously [27].

Western blot

Western blot was used to detect the protein expression. The Western blots detecting the VEGF, phospho-ERK, phospoh-JNK, phospho c-Jun, total c-Jun and α-tubulin protein expressions were processed as described previously [27].

Gel-shift analysis

Electrophoretic mobility-shift assay (EMSA) was performed to detect the formation of complexes between cellular proteins and the AP-1 transcription element. The gel-purified, double-strand DNA fragments including the standard wild type AP-1 and 2 AP-1 sites consisted in VEGF promoter (standard wild type AP-1: 5′-cgcttgatgactcagccg-gaa-3′; VEGF promoter, −1227∼−1215: 5′-ctat-gagtctggg-3′; −937∼−925: 5′-cactgactaaccc-3′) were radio-labeled by the reaction mixed with γ-^{32}P-ATP, T4 polynucleotide kinase in 1× PNK buffer at 37 °C for 30 min. The nuclear cell fractions were prepared as described previously [28]. Five-microgram aliquots of either nuclear fraction were incubated with 50,000 cpm of DNA probe at room temperature for 20 min as described previously [28]. Reaction mixtures were electrophoresed through 6% non-denaturing polyacrylamide gels containing 0.25× Tris–borate–EDTA (TBE) at 150 V for 1.5 h at 4 °C. Gels were dried and the radioactive signals were visualized with autoradiography.

Luciferase reporter DNA construction, transient transfection, and luciferase assay

Two plasmid constructs were used in the luciferase reporter assay. One is the VEGF promoter (−1828 ∼ −984, PstI to NheI) conjugated to the luciferase gene of pGL2-Basic Vector [29]. The other is the di-repeats of AP-1 site (TGACTCAT-GACTCA) conjugated to the luciferase gene of p19Luc Vector (obtained from Dr. Tze-Sing Huang). The transfection experiments were performed using the Lipofectin reagent following the manufacturer's protocol (Invitrogen). Briefly, luciferase construction (1 μg) and Renilla luciferase vector (1 μg) were mixed with 100 μl of serum free medium in one tube. Another tube contained the 10 μl of lipofectin mixed with 100 μl of serum free medium. Both mixtures were stood at room temperature for 40 min. Then, both mixtures were mixed together and stood for further 15 min at room temperature. Finally, the mixture was added into the cells in 60-mm plate that were changed with 0.8 ml serum free medium before. After 6 h of transfection, the cells were replaced with 2 ml fresh medium for overnight. After HBO treatment, luciferase assays were performed according to the protocol of Luciferase assay kit (Promega, Madison, WI). Briefly, treated cells were lysed using the provided lysis buffer. After centrifuged at 18,000 *g* for 5 min, the supernatants were collected. Twenty microliters of lysates were added to 100 μl of

luciferase substrate (LARII). The arbitrary units of luminescence were detected with a Luminometer (TD-20/20, Turner Designs Inc.). After reading the luminescence of excited by luciferase, immediately added 100 μl of STOP reagent provided in the assay kit and vortex to mix. Reapplied the sample to the luminometer to detect the luminescence excited Renilla luciferase.

Statistical tests

All values are expressed as the mean ± SD of triplicate. Differences between the groups were calculated by ANOVA with *post hoc* analysis (LSD). A value of $p < 0.05$ was considered significant.

Results

HBO induces the translation and transcription of VEGF in HUVECs

Initially, we investigated the VEGF protein expression in HUVECs treated with 1 or 2.5 ATA of pure oxygen for 8 h. Western blot showed that VEGF protein expression was significantly induced obviously with 2.5 ATA of pure oxygen (about 3 fold than normoxia), but was not induced with 1 ATA of pure oxygen (Figure 1a). Furthermore, we treated HBO (2.5 ATA of pure oxygen) on HUVECs with different time intervals, and found that the VEGF protein was induced from 6 to 12 h of HBO treatment. The highest level (3–4 fold) of VEGF induced by HBO was at 6–8 h of HBO treatment (Figure 1b). Northern blot also showed that the VEGF mRNA was induced by HBO with 2.5 ATA after 4 h, however, pure oxygen in normal pressure (1 ATA) did not alter VEGF mRNA expression after 4 h (Figure 2a). We tried different time intervals of HBO (2.5 ATA) treatment (from 2 to 8 h) to observe the time course of VEGF mRNA expression affected by HBO. The level of VEGF mRNA expression in the cells treated with HBO was significantly higher than normoxia in each time point from 2 to 8 h (Figure 2b). The VEGF mRNA expression was induced by HBO time dependently, and was up regulated to the highest level (2–3 fold) at about 4–6 h of HBO treatment (Figure 2b). From these data, we suggest that HBO not only induces VEGF mRNA expression but also VEGF protein in HUVECs. However, pure oxygen does not induce VEGF mRNA expression, and neither does VEGF protein.

HBO induces the VEGF expression through VEGF receptor 2, tyrosine kinases, MEK/ERK, and SAPK/JNK activation

Since the VEGF was induced by HBO in endothelial cells, we further investigated the signal pathway that might be involved in the induction of VEGF by HBO. We pretreated HUVECs with several signal transduction pathway inhibitors 30 min before HBO treatment. These include: N(omega)-nitro-L-arginine methyl ester (L-NAME) (NOS inhibitor); SU1498 (KDR, VEGF receptor 2 inhibitor); PD98059 (MEK/ERK inhibitor); Wortmannin, (PI-3 kinase inhibitor); Genistein (tyrosine kinase inhibitor); Go6976 (PKC inhibitor); SP600125 (JNK inhibitor). After 30 min of these inhibitors pretreatment and 4 h of HBO (2.5 ATA) treatment, the endothelial cells were put to analyze the VEGF mRNA expression by Northern blot. As shown in Figure 3, the VEGF mRNA was induced after 4 h of HBO treatment. The induction of VEGF mRNA by HBO was significantly blocked by the action of SU1498 (10 μM), PD98059 (50 μM), Genistein (40 μM) and SP600125 (25 μM). The VEGF mRNA induced by HBO was though significantly but partially inhibited by L-NAME (3 mM) and wortmannin (50 nM). However, the PKC inhibitor (Go6976, 16 nM)) did not alter the VEGF mRNA expression induced by HBO. All the inhibitors did not lose their specific inhibition ability, since we individually checked all these inhibitors' pharmaceutical function (data not shown). These results indicate that the VEGF mRNA expression induced by HBO may be correlated with the activation of VEGF receptor 2, tyrosine kinases, MEK/ERK, and SAPK/JNK activation.

HBO induces the VEGF expression through ERK, JNK and c-Jun activation

From the results described above, we suggest that HBO induces VEGF expression through simultaneous effect from ERK and JNK activation. We checked both the phosphorylation type of

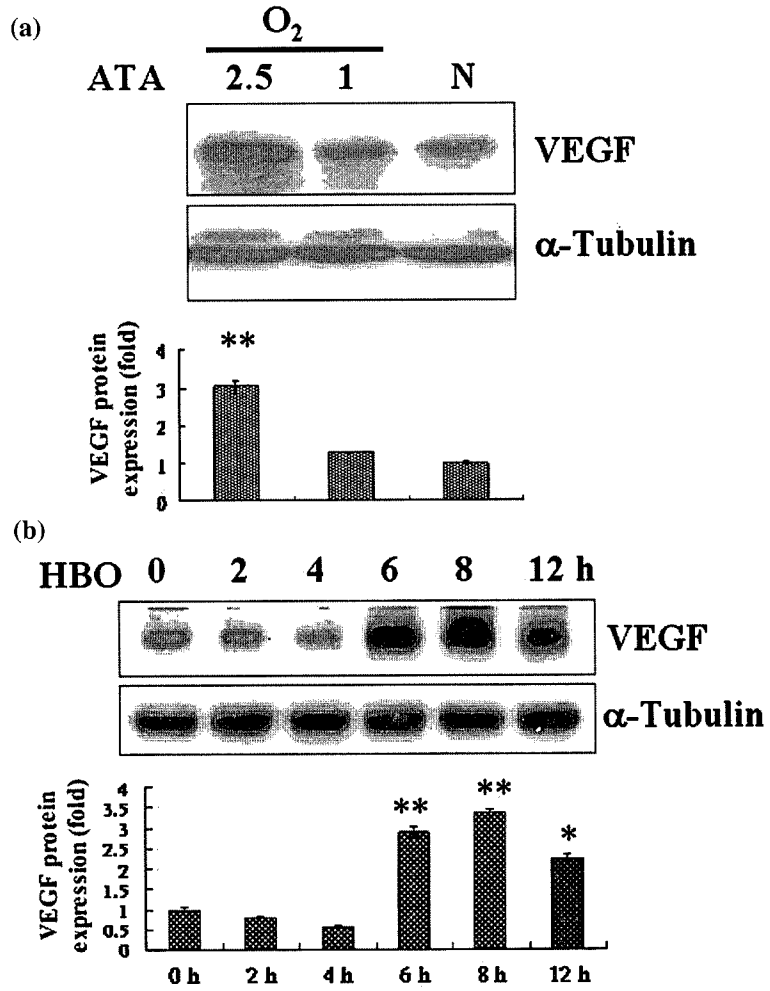


Figure 1. The VEGF protein expressions of HUVECs treated with pure oxygen. (a) Western blot of VEGF protein expressions in HUVECs treated by normoxia (N) and oxygen with 1 or 2.5 ATA for 8 h. The value of each VEGF signal was normalized with the value of its corresponding α-tubulin. Finally, all the ratios normalized with the ratio of normoxia. **: $p < 0.01$ vs. normoxia, $n = 3$. (b) Time course of VEGF protein level caused by HBO (oxygen, 2.5 ATA) in HUVECs. The value of each VEGF signal was normalized with its corresponding α-tubulin value. Then, all the ratios normalized with the ratio of control 0 h. *: $p < 0.05$; **: $p < 0.01$, HBO vs. control, $n = 3$.

ERK and JNK by Western blot. After HBO treatment, both the phospho-ERK and the phospho-JNK were significantly elevated in 15 min (Figure 4a). These results indicate that HBO immediately and simultaneously induces phospho-ERK and phospho-JNK in endothelial cells within 15 min.

In the past, it has been reported that VEGF promoter contains at least three AP-1 sites [20], and the AP-1 is not only regulated through JNK and c-Jun activation, but also regulated through ERK activation [25]. C-Jun is a component of the transcription factor AP-1 that binds and activates transcription at TRE/AP-1 elements. The trans-acting ability of c-Jun is activated by phosphorylation at Ser 63 and Ser 73 by JNK [21, 22]. We further investigated the effect of HBO on the activation of c-Jun /AP-1 in endothelial cells. We analyzed the phospho-c-Jun (Ser 73) and total c-Jun protein by Western blot. After normalized with total c-Jun protein expression, we found that the phospho-c-Jun (Ser 73) was significantly up regulated after 1 h of HBO treatment (Figure 4b). From these results, we suggest that HBO induces the ERK, JNK and c-Jun phosphorylation, and thereafter, induces the c-Jun/AP-1 activation.

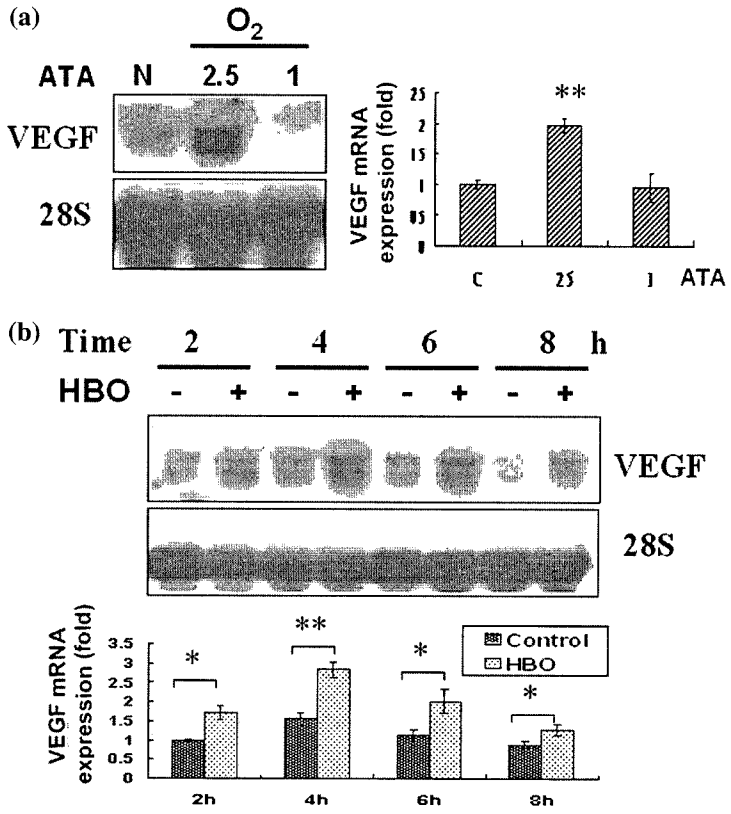


Figure 2. The VEGF mRNA expressions of HUVECs treated with pure oxygen. (a) Northern blot of VEGF mRNA expressions in HUVECs treated by normoxia (N) and oxygen with 1 or 2.5 ATA for 4 h. The value of each VEGF signal was normalized with the value of its corresponding 28S. Finally, all the ratios normalized with the ratio of normoxia. **: $p<0.01$ vs. normoxia, $n=3$. (b) Time course of VEGF mRNA expressions in HUVECs treated by normoxia (−) and HBO (oxygen, 2.5 ATA) (+) in HUVECs. The value of each VEGF signal was normalized with its corresponding 28S value. Then, all the ratios normalized with the ratio of control 2 h. *: $p<0.05$; **: $p<0.01$, HBO vs. each time interval of control, $n=3$.

HBO induces nuclear binding ability on AP-1 sites through ERK and JNK pathways

From the EMSA results, we found that HBO induced the nuclear binding ability of AP-1 in endothelial cells starting from 30 min to 2 h (Figure 5a). A shift signal (super shift) was present when the sample was co-incubated with c-Jun antibody (Figure 5b). However, the super shift of AP-1 binding was not found when the sample was co-incubated with c-Fos antibody (Figure 5b). To prove that the signal of the binding induced by 1 h of HBO was real AP-1, we replaced the probe with a mutant AP-1 with just one nucleotide change. The AP-1 binding was nearly lost when the nuclear extract was incubated with the mutant probe (Figure 5b). We also checked the AP-1 binding activity of the endothelial cells pretreated with MEK/ERK inhibitors, PD98059 (50 μM) and U0126 (25 μM), and SAPK/JNK inhibitor, SP600125 (25 μM) for 30 min before 1 h of HBO treatment. All three inhibitors blocked the AP-1 binding ability induced by HBO (Figure 5c). These results indicate that HBO induces AP-1 binding ability through simultaneous activation of ERK and JNK pathways. Since it has been reported that VEGF promoter contains several AP-1 binding sites, we searched the DNA sequence of the VEGF promoter based on the conserved sequence of AP-1, TGANT(C/A)NN [20]. Primarily, we selected six sites of VEGF promoter which the DNA sequence was homology to the AP-1 conserved sequence, TGANT(C/A)NN (20). These 6 sites are individually located at −2224∼2212: 5′-gagtgactggtga-3′; −1521∼−1509: 5′-gactgactggcct-3′; −1227∼−1215: 5′-ctatgagtctggg-3′; −937∼−925: 5′-cactgactaaccc-3′; −542∼−530: 5′-gggtgaatggagc-3′; −490∼−478: 5′-gggtgagtgagtc-3′. We tested the

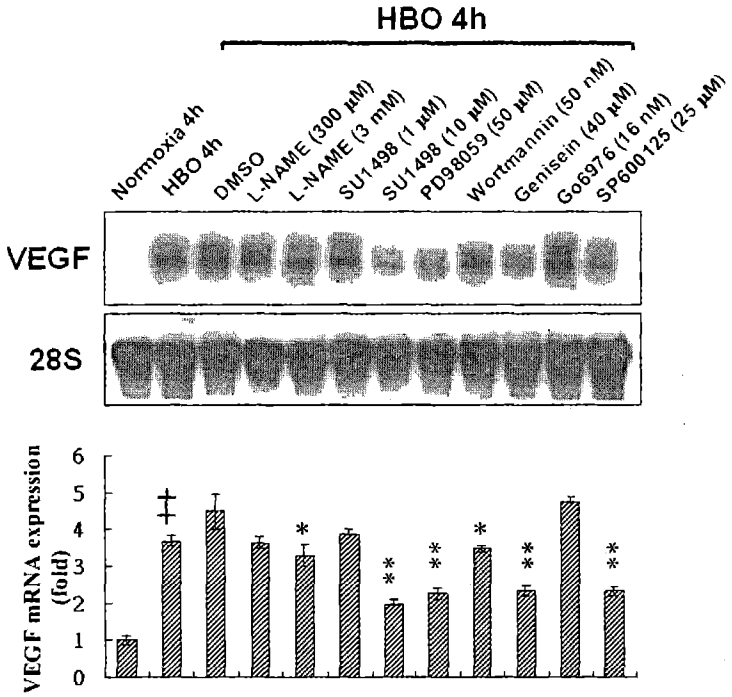


Figure 3. The VEGF mRNA expressions in HBO-induced HUVECs pretreated with inhibitors. The VEGF mRNA expressions in the HUVECs treated with various inhibitors for 30 min before the treatment of HBO (2.5ATA) for 4 h. The value of each VEGF signal was normalized with the value of its corresponding 28S. Finally, all the ratios normalized with the ratio of normoxia 4 h. ‡: $p<0.01$ vs. normoxia 4 h, $n=3$. ⁑: $p<0.01$ vs. DMSO in HBO 4 h, $n=3$. *: $p<0.05$ vs. DMSO in HBO 4 h, $n=3$.

nuclear binding ability to these sites by PMA, and found two of the six sites (−1227∼−1215, −937∼−925) that the binding abilities were enhanced by the PMA treatment. Also, the PMA induced binding abilities of these two sites were competitively inhibited by non-radio-labeled (cold) standard wild type AP-1. The nuclear binding ability to each of the two sites was tested in the endothelial cells treated with HBO. We found that the binding abilities to these two sites were all induced by 30 min of HBO (Figure 5d). From these results, we confirmed that at least two sites were AP-1 transcription elements, and HBO induced the nuclear binding ability to these 2 AP-1 transcription elements in VEGF promoter.

HBO induces the VEGF- and AP-1 promoter driving luciferase activity

In order to confirm our finding that HBO induces VEGF through activation of AP-1, we used the VEGF promoter (−984 ∼ −1828) and di-repeats of AP-1 luciferase reporter constructs to detect the VEGF promoter and AP-1 activity in the cells treated by HBO. Since the transfection efficiency of the reporter constructs in HUVECs is low, we used the lung cancer cell line, CL1-0, as a host for the reporter constructs transfection. We found that 1 h of HBO increased both the VEGF promoter (−984 ∼ −1828) and di-repeats AP-1-luciferase activity in CL1-0 cells with about 3- and 6-fold, respectively (Figure 6a, b). From the luciferase assays in CL1-0, though not in endothelial cells, the results are consistent with the conclusion that HBO induces VEGF expression through VEGF promoter and AP-1 trans-activation in endothelial cells.

HBO induces the c-Jun mRNA expression through ERK and JNK pathways

We checked the c-Jun mRNA expression by Northern blot, since the c-Jun transcription is also regulated by AP-1 element. Just as VEGF, the c-Jun mRNA was significantly up regulated after 4 h of HBO treatment (Figure 7a). Furthermore, we

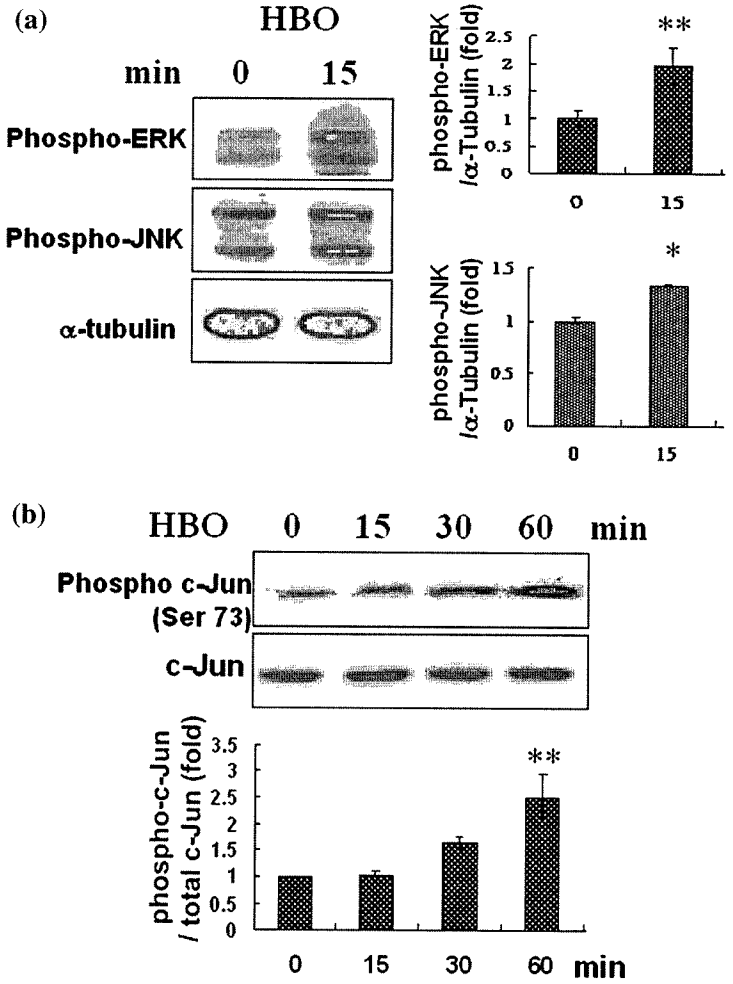


Figure 4. The phospho-ERK, phospho-JNK and phospho-c-Jun expressions in HUVECs treated with HBO. (a) phospho-ERK and phospho-JNK protein expressions in HUVECs treated with HBO (2.5 ATA) for 15 min. The value of each phospho-ERK or phospho-JNK signal was quantified and normalized with its corresponding α-tubulin. Finally, all the ratios normalized with the ratio of control 0 h. *: $p<0.05$; **: $p<0.01$, HBO vs. control 0 h, $n=3$. (b) Time course of phospho-c-Jun and total c-Jun expressions in HUVECs treated by HBO (2.5 ATA). The value of each phospho-c-Jun was normalized with the corresponding total c-Jun. Finally, the ratios were normalized with the ratio of control 0 h. **: $p<0.01$, HBO 60 min vs. control 0 h, $n=3$.

investigated if the c-Jun mRNA expression was regulated by ERK or JNK when endothelial cells were treated with HBO. We pretreated HUVECs with various inhibitors, including PD98059, U0126 (MEK/ERK inhibitor) and SP600125 (JNK inhibitor), 30 min before HBO treatment. After HBO treatment for 4 h, the cells were put to analyze the c-Jun mRNA expression by Northern blot. The induction of c-Jun mRNA by HBO was significantly blocked by PD98059 (50 μM), U0126 (25 μM) and SP600125 (25 μM) (Figure 7b). These results indicate that the HBO inducing c-Jun mRNA is through the activation of ERK and JNK pathways.

Discussion

Molecular oxygen plays a central role in the reparative process and is one of the critical nutrients of the wound [6]. Collagen synthesis, matrix deposition, angiogenesis, epithelialization and bacteria killing all require molecular oxygen during the reparative process. Angiogenesis and epithelialization rate are also oxygen-dependent. Oxygen supply controls the rate of epithelialization in normal and ischemia wounds when oxygen is administered 1–2 atmospheres absolute (ATA) [30]. Angiogenesis is driven by a gradient of

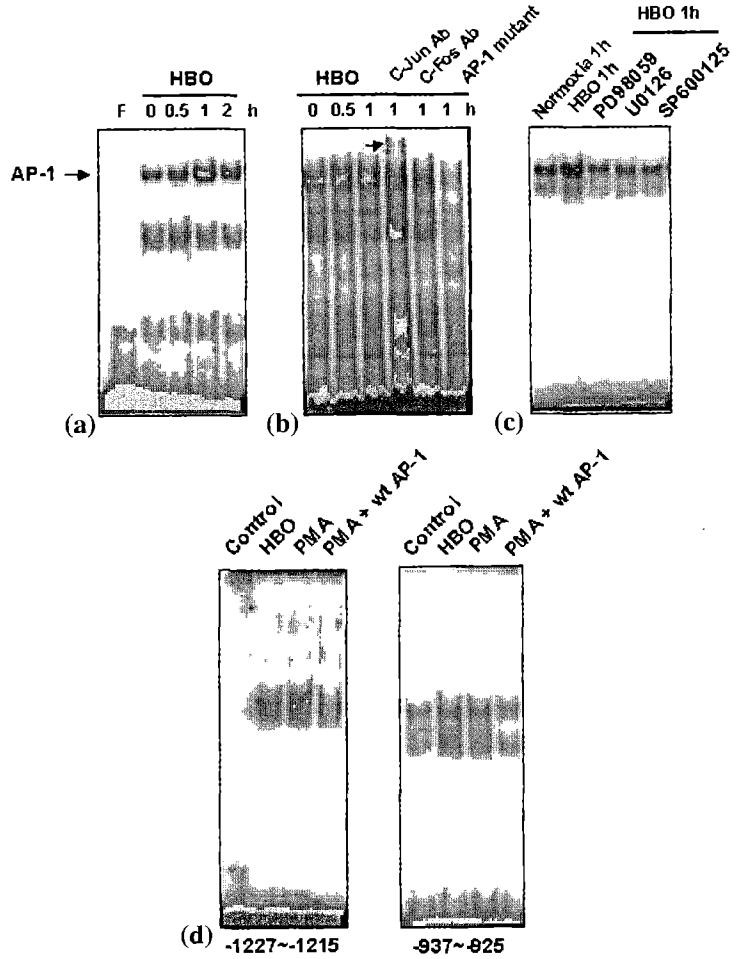


Figure 5. The AP-1 nuclear binding ability in HUVECs treated with HBO. (a) Time course of the AP-1 (5′-cgcttgatgactcagccggaa-3′) nuclear binding ability induced by HBO (2.5 ATA). (b) The AP-1 nuclear binding ability in different time intervals of HBO treatment (2.5 ATA), and the appearance of super shift band (arrowhead) when the reaction mixture combined with 1 μl of c-Jun or c-Fos antibody. Mutant AP-1 probe (5′-cgcttgaggactcagccggaa-3′) was as a negative control to confirm that the signal induced by HBO is real AP-1. (c) The AP-1 nuclear binding ability induced by 1 h of HBO treatment (2.5 ATA) or that pretreated with various inhibitors, PD98059 (50 μM), U0126 (25 μM) and SP600125 (25 μM) for 30 min. (d) Two AP-1 sites in VEGF promoter, −1227 ~ −1215 and −937 ~ −925(sequences were described in Materials and methods), were as probes for nuclear binding ability assay in endothelial cells treated with normoxia (control), HBO (30 min) or PMA (30 min). The non-radio-labeled (cold) standard wild type AP-1 was added to the reaction mixture to competitively block the binding ability of AP-1 site.

oxygen where by high arterial PO_2 drives angiogenesis into hypoxic spaces [31]. Since the angiogenesis is directed by endothelial cell and by the factor of VEGF, in this study, we initially investigated the regulation of VEGF expression in HUVECs treated with pure oxygen with varied atmospheres (1 or 2.5 ATA). The VEGF expression was induced by the increase of atmosphere of oxygen (2.5 ATA) both in mRNA and protein levels. However, pure oxygen (1 ATA) neither increased VEGF in the protein nor in the mRNA levels in the endothelial cells. These results suggest that the induction of VEGF expression is correlated with the pressure of oxygen, and support the theory that HBO is more effective than oxygen in normal atmosphere on wound healing process through inducing angiogenesis.

The effect of HBO on stimulating angiogenesis and endothelial cells growth was certified by either *in vivo* or *in vitro* models [8, 12–14]. It has also been investigated that VEGF was induced by HBO in a rat wound model [32]. However, in our previous study using cultured endothelial cell model, we did not find VEGF induced by HBO

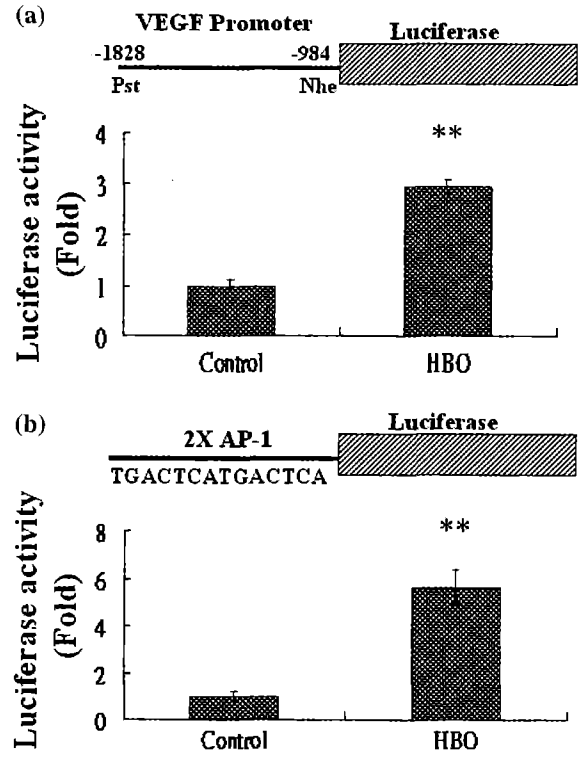


Figure 6. The luciferase reporter assays of VEGF promoter and AP-1 driving luciferase activity induced by HBO in lung cancer cells, CL1-0. Lung cancer cells, CL1-0, co-transfected (a) VEGF promoter (−1828 ~ −984)- luciferase construction or (b) di-repeats of AP-1-luciferase construction with Renilla luciferase construction. After transfection and recovery, cells were put to HBO treatment or control (normoxia) for 1 h, and were put to detect the luminance excited by luciferase and Renilla luciferase. The values of luminance excited by luciferase were normalized with the values of luminance excited by Renilla luciferase. Finally, the ratio of HBO treatment was normalized with the ratio of control (normoxia). $^{**}p < 0.01$, HBO vs. control. $n = 3$.

in one and a half hour [19]. Since the period of 90 min of HBO treatment may be not long enough for detecting some genes activation, the results that HBO induced VEGF and other angiogenesis factors might be missed when we observed the results just within 90 min of HBO treatment. In this study, we treated HUVECs with HBO from 2 to 12 h and exactly found that the VEGF was induced by HBO through both transcription and translation steps. In this study, we demonstrated that one of the mechanisms involved in the angiogenesis induced by HBO is through the VEGF induction in endothelial cells. In wound healing process, endothelial cells may be involved in the process of angiogenesis and are the source of releasing VEGF. However, VEGF and some other angiogenic factors may also be derived from some other cells, such as macrophage and fibroblast. In fibroblast cells, it has been studied that basic fibroblast growth factor was increased 1 day after the cells were treated with HBO for 90 min. However, in the same study, VEGF was only slightly induced but not significantly in fibroblast cells after HBO treatment [33]. There was no report investigating the alteration of angiogenic factors in macrophage treated with HBO. But one report investigated that VEGF was induced in macrophage treated with H_2O_2 [34]. In this study, the direct evidence of HBO inducing VEGF expression in endothelium may be consistent with the finding that VEGF is involved in angiogenesis process induced by HBO in rat model of wound healing [32].

In order to know the mechanism involved in the induction of VEGF expression by HBO, we pretreated various inhibitor of signal pathways in the endothelial cells treated with HBO. Our results showed that SU1498 (10 μM), PD98059, Genistein and SP600125 significantly blocked the induction of VEGF mRNA expression by HBO. It seems that HBO induces VEGF through VEGF receptor 2, tyrosine kinases, MEK/ERK and JNK activation. SU1498 is an inhibitor of VEGF receptor 2. However, it has been reported that SU1498 stimulates accumulation of phosphorylated ERKs in HUVECs and in human aortic endothelial cells, but the inhibitor blocked the kinase activity of phospho-ERK both in a direct assay and in immunoprecipitates. These findings reveal a novel and unique way in which MAPK signaling pathway may be blocked by SU1498 in human endothelial cells [35]. The effects of genistein on neovascularization, VEGF, and hypoxia inducible factor 1α (HIF-1α) protein expression in a mouse model of oxygen-induced retinopathy were studied. Genistein markedly inhibited the induction of HIF-1α expression and relative-hypoxia-elicited VEGF expression in a dose-dependent manner in oxygen-reared animals when compared to room-air-reared animals. These results indicated that the inhibition of VEGF and HIF-1α protein expression by genistein may partly account for its effect on retinal neovascularization *in vivo*, and genistein could be an effective agent in the prevention and treatment of ocular neovascularization [36]. The reason and result for that SU1498 and genistein

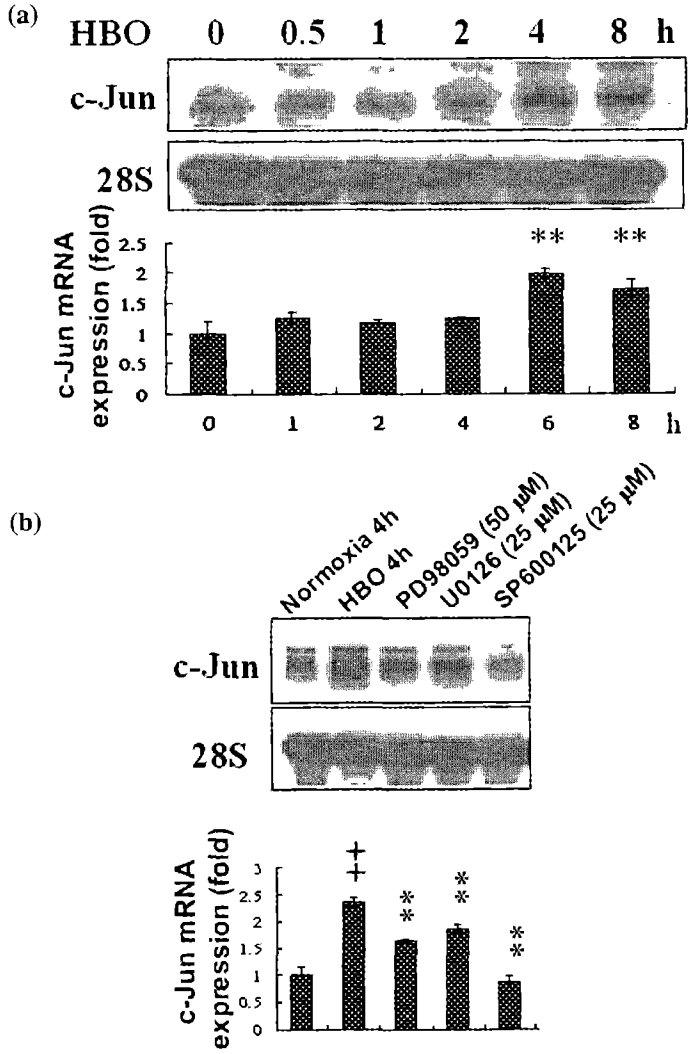


Figure 7. The c-Jun mRNA expression in HUVECs treated with HBO, and the c-Jun mRNA expression pretreated with inhibitors in HUVECs before HBO treatment. (a) Time course of c-Jun mRNA expressions in HUVECs treated by HBO (oxygen, 2.5 ATA). The value of each c-Jun mRNA signal was normalized with its corresponding 28S value. Then, all the ratios normalized with the ratio of control 0 h. **: $p<0.01$, HBO vs. control, $n=3$. (b) The c-Jun mRNA expressions in the HUVECs treated with various inhibitors for 30 min before the treatment of HBO (2.5 ATA) for 4 h. The value of each c-Jun signal was quantified and normalized with the value of corresponding 28S. Finally, all the ratios normalized with the ratio of normoxia 4 h. ‡: $p<0.01$ vs. normoxia 4 h; ⁂: $p<0.01$ vs. HBO (DMSO) 4 h, $n=3$.

blocked the HBO inducing VEGF expression are not clear and worth to be further investigated.

By binding to its receptors, VEGF initiates the signaling cascades leading to NO production and angiogenic activation of endothelial cells. However, there are abundant evidences that nitric oxide (NO) is an effective molecule mediating the activity of VEGF. Recent data show that NO induces VEGF synthesis in numerous cell types, including vascular smooth muscle cells, macrophages, keratinocytes, and tumor cells. NO enhances VEGF production by augmenting its expression through activation of Akt kinase, followed by induction of several transcription factors, of which stabilization of HIF-1 is the critical step. The effect of NO on VEGF production is also mediated by heme oxygenase, an enzyme generating carbon monoxide, which appears to stimulate VEGF release [37]. In our previous result, we also found that HBO inducing Ang 2 might be mediated through NO

signal [19]. In this study, since L-NAME partially inhibited the induction of VEGF from HBO, the NO pathway may partially involve in the initial induction of VEGF by HBO. Since wortmannin partially inhibited the VEGF induced by HBO, the PI-3 kinase/Akt pathway may also be the same constitution as NO pathway involved in the regulation VEGF when HBO treatment. It has been studied that NO signal activation is correlated with PI-3 kinase/Akt activation [38]. The induction of VEGF expression through NO and PI-3 kinase/Akt pathway will be identified in the future. Our results exclude the PKC pathway involved in the VEGF induced by HBO, since the VEGF induction was not inhibited by the action of PKC inhibitor, Go6976.

Our study focused on the MEK and JNK pathways that may be involved in the VEGF expression induced by HBO. In this study, we demonstrated that both of the phospho-ERK and phospho-JNK were induced by HBO in 15 min of HBO treatment. We further checked the downstream signal of JNK activation, phospho-c-Jun, and found that the phosho-c-Jun was also induced in 1 h of HBO treatment. The c-Jun phsphorylation may induce the transcription of the genes whose promoters contain AP-1 site, such as VEGF [20] and c-Jun itself [39]. Other than VEGF, our study also confirmed that c-Jun mRNA expression was induced after 4–8 h of HBO treatment, and the induction of c-Jun mRNA expression was blocked by the action of ERK and JNK inhibitors. The AP-1 binding ability was induced between 30 min and 2 h of HBO treatment, and the enhancement of AP-1 binding ability was also inhibited by the action of ERK and JNK inhibitors. Our study demonstrated that the VEGF expression induced by HBO was through c-Jun/AP-1 activation and simultaneously regulated through ERK and JNK activation.

In order to confirm that HBO induces VEGF gene expression through the AP-1 activation that exists in VEGF promoter, we search the sequence of 2362 bp of the VEGF promoter (from −1 to −2362). We found that 2 sites (−1227∼−1215: 5′-ctatgagtctggg-3′; −937∼−925: 5′-cactgactaaccc-3′) not only were homology to the AP-1 conserved sequence, but also were identified as enhancement of nuclear binding after the cells treated with phorbol 12-myristate 13-acetate (PMA). Furthermore, the induction binding ability of these two sites by PMA were competitively inhibited by standard wild type AP-1 DNA fragment (5′-cgcttgatgactcagccggaa-3′). These two sites were further put to analyze the endothelial cells treated with HBO, and found that all the two sites were enhanced binding by the nuclear extract of HBO treated cells. In our study, we also tested both the VEGF promoter (−984∼−1828, containing one AP-1 site, −1227∼−1215) and di-repeats of AP-1-luciferase reporter assays in endothelial cells treated with HBO. The low transfection efficiency of the reporter constructions in HUVECs made us hardly to get the significant result. However, when we used the lung cancer cell line, CL1-0, as a host for the VEGF promoter- and AP-1-luciferase expression, we found that HBO increased both the VEGF promoter- and AP-1-luciferase activity in CL1-0 cells. From the gel shift assays and luciferase assays, the results are consistent with the conclusion that HBO induces VEGF expression through VEGF promoter and AP-1 transactivation in endothelial cells.

Our study also checked the c-Fos, the other AP-1 transacting protein that heterodimerized with c-Jun [40], induced by HBO. Our result revealed that the c-Fos expression was not influenced by HBO (data not shown). The result of c-Fos was also confirmed by the EMSA assay, which the super-shift band was only found in the sample with c-Jun antibody but not found with c-Fos antibody. Our results suggest that c-Jun, but not c-Fos, is the main AP-1 binding protein activated by HBO, and hence induces the downstream genes expression such as VEGF and c-Jun.

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