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Repetitive Hyperbaric Oxygen Treatment Attenuates Complete Freund's Adjuvant-Induced Pain and Reduces Glia-Mediated Neuroinflammation in the Spinal Cord

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Abstract: Hyperbaric oxygen (HBO) therapy is reported to attenuate pain in both clinical pain conditions and animal pain models, but the underlying mechanism remains to be investigated. Here, we show that 7 daily 60-minute HBO (100% oxygen, 2 atmosphere absolute) treatments effectively and persistently inhibited heat hyperalgesia, mechanical allodynia, and paw edema induced by peripheral injection of complete Freund's adjuvant (CFA). Five daily 60-minute HBO treatments also produced a prolonged reversal effect of the ongoing inflammatory pain. Furthermore, such an HBO treatment reduced CFA-induced activation of glial cells, phosphorylation of mitogen-activated protein kinases, and production of a variety of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin-1 beta [IL-1β], and interleukin-6 [IL-6]) and chemokines (monocyte chemoattractant protein-1 [MCP-1], keratinocyte-derived chemokine [KC], and IFN-gamma-inducible protein 10 [IP-10]) in the spinal cord. HBO treatment also decreased lipopolysaccharide-induced mRNA expression of these cytokines and chemokines in primary cultures of astrocytes and microglia. In addition, the mRNA expressions of IL-1β, IL-6, MCP-1, KC, and IP-10 in the inflamed paw skin were decreased by HBO. Taken together, these data suggest that HBO treatment is an effective therapy for inflammatory pain in animals. The inhibition of the neuroinflammation that is mediated by glial cells and inflammatory mediators may, at least in part, contribute to the antinociceptive effect of HBO therapy.

***Perspective:** Our results suggest that repetitive HBO treatment attenuates CFA-induced pain and reduces glial activation and inflammatory mediators' production. These findings provide evidence of the antinociception effect of HBO on inflammatory pain and characterize some of the underlying mechanisms.*

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***Key words:** Hyperbaric oxygen treatment, astrocytes, microglia, cytokines, chemokines, mitogen-activated protein kinase, inflammatory pain, antinociception.*

Hyperbaric oxygen (HBO) therapy is a clinical therapy that involves administering 100% oxygen at a pressure higher than atmospheric pressure at sea level for a prescribed amount of time. Clinical observations are increasingly indicating that HBO appears to be effective in some chronic pain conditions such as headache,[9] complex regional pain syndrome,[26] myofascial pain syndrome,[25] and idiopathic trigeminal neuralgia.[18] In experimental animals, HBO treatment attenuates neuropathic pain induced by spinal nerve ligation or chronic constriction injury (CCI) of the sciatic nerve[28,44] and acute inflammatory pain induced by peripheral or intra-articular injection of carrageenan,[51] or intraperitoneal injection of glacial acetic acid.[6] These reports indicate a potential role of HBO in antinociception. However, the effects of HBO on chronic inflammatory pain and the underlying mechanisms remain unclear.

HBO has been shown to regulate some aspects of host defense[3] and to inhibit macrophage function and

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inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β),[2,28,39] suggesting that some beneficial effects of HBO may be attributable to an altered inflammatory-like process. It has been recognized that the nervous system exhibits inflammatory processes in response to injury, infection, or disease. In addition, inflammation has been implicated to be a driving force for the pathogenesis of chronic pain by producing multiple inflammatory mediators such as prostaglandin, proinflammatory cytokines (eg, TNF-α and IL-1β), and chemokines (eg, monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein-1α [MIP-1α]) in inflamed or damaged tissues.[42,52] In the central nervous system, inflammatory mediators are also produced by glial cells (eg, microglia and astrocytes) and involved in regulation of the neuronal excitability by glial-neuronal interaction.[16,55] Accumulating evidence supports an important role of spinal glial cells and inflammatory mediators in promoting chronic pain.[12,37,40,48] However, whether HBO could reduce the inflammation in the peripheral tissue and regulate the activity of glial cells in the spinal cord under chronic pain conditions remains to be investigated.

In recent years, an increasing list of signaling molecules in glial cells has been implicated in persistent pain.[15,17] The mitogen-activated protein kinases (MAPKs), which include 3 major members—extracellular signal regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)[23]—are activated in spinal glia after nerve injury or tissue injury and play an important role in chronic pain by signaling to the inflammatory mediators.[22] The MAPKs are not only activated by inflammatory mediators, but also increase the synthesis of multiple inflammatory mediators.[15] Recent studies have demonstrated that inhibition of MAPKs attenuates chronic inflammatory pain.[11]

In this study, we examined the effect of repetitive HBO treatment on complete Freund's adjuvant (CFA)-induced inflammatory pain and paw edema. We also explored the possible mechanisms of HBO by checking the activation of glial cells and MAPKs in the spinal cord, as well as the production of inflammatory mediators in the spinal cord, primary cultured glial cells (astrocytes and microglia), and inflamed skin.

Methods

Animals and Peripheral Pain Model

Male adult ICR mice (20–30 g) were purchased from the Experimental Animal Center of Nantong University (Nantong, China). Mice were housed in plastic cages and maintained on a 12:12 hour light/dark cycle under conditions of 23 ± 1°C with food and water available. All surgical and experimental procedures were reviewed and approved by the Animal Use and Care Committee for Research and Education of Nantong University. Animal treatments were performed according to the Guidelines of the International Association for the Study of Pain. Peripheral inflammation was induced by intraplantar injection of CFA (20 μL, Sigma-Aldrich, St. Louis, MO) in the left hind paw under brief anesthesia with isofluorane.

Hyperbaric Oxygen Treatment

The animals were randomly assigned to 4 groups: HBO group (100% oxygen, 2 ATA [atmosphere absolute]), hyperbaric air (HBA) group (air, 2 ATA), pure oxygen group (100% oxygen, 1 ATA), and control group (air, 1 ATA). For hyperbaric treatment, the animals received 100% oxygen (HBO) or air (HBA) at a pressure of 2 ATA in an animal hyperbaric monochamber for 60 minutes. The compression and decompression were performed within 10 minutes. Therefore, each treatment lasted in total approximately 80 minutes. The parameters of pure oxygen treatment used were 1 ATA in 100% O_2 for 80 minutes. The animals in the control group were placed inside the hyperbaric treatment chamber for 80 minutes and did not receive any treatment. To examine whether HBO could prevent inflammatory pain, the animals were treated with HBO from day 0 to day 6 after CFA injection for 7 consecutive days. Furthermore, to examine whether HBO could reverse established inflammatory pain, the animals were treated with HBO from day 3 to day 7 after CFA injection for 5 consecutive days.

Behavioral Analysis

Animals were habituated to the testing environment daily for at least 2 days before baseline testing. The room temperature and humidity remained stable for all experiments. The volume of hindpaws was measured via water displacement utilizing a plethysmometer (Ugo Basile, Camerio, VA, Italy). For heat hyperalgesia, animals were put in a plastic box placed on a glass plate, and the plantar surface was exposed to a beam of radiant heat through a transparent glass surface (IITC model 390 Analgesia Meter; Life Science, Woodland Hills, CA).[19] The baseline latencies were adjusted to 12 to 15 seconds with a maximum of 20 seconds as cutoff to prevent potential injury. The latencies were averaged over 3 trials, separated by a 5-minute interval. For mechanical allodynia, animals were placed on a wire mesh floor and confined underneath individual overturned plastic boxes. Mechanical allodynia was assessed using 2 von Frey filaments with bending forces of .008 g and .02 g (Stoelting Co, Wood Dale, IL). In ascending order of force, each von Frey filament was applied 20 times (2 sets of 10 stimulations were separated by approximately 10 minutes to decrease possible sensitization). Withdrawal responses to each of the von Frey filaments were counted. The response percentage of paw withdrawals out of 20 stimuli was calculated.[4]

Cell Culture and Treatment

Primary microglial and astrocytes cultures were prepared from cerebral cortexes of neonatal mice (postnatal day 1, P1).[16,21,57] The cerebral hemispheres were isolated and transferred to ice-cold Hank's buffer

(Invitrogen, Carlsbad, CA), and the meninges were carefully removed. Tissues were then minced into 1-mm pieces, triturated, filtered through a 100-μm nylon screen, and collected by centrifugation at 3,000 g for 5 minutes. For microglial culture, the cell pellets were dispersed with a pipette and resuspended in a medium containing 10% fetal bovine serum in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen). After trituration, the cells were filtered through a 10-μm screen, plated into 75-cm^2 flasks. The medium was replaced twice a week. After 12 to 14 days, the flasks were shaken on a rotary shaker at 220 rpm for 4 hours. The resulting cell suspension, rich in microglia (>98%), was placed in culture dishes in which the cells adhered after 30 minutes at 37°C. The cells were treated by lipopolysaccharide (LPS) after 24 hours. For astrocytes culture, the cell pellets were resuspended in a medium containing 10% fetal bovine serum in low-glucose DMEM. After filtration through a 10-μm screen, the cells were plated into 6-well plates at a density of 2.5×10^5 cells/cm^2 and cultured for 10 to 12 days. The medium was replaced twice a week. Once the cells were grown to 95% confluence (10–12 days), .15 mM dibutyryl cyclic adenosine monophosphate (cAMP) (Sigma-Aldrich) was added to induce differentiation. The cells can be used 3 days later.

When the cells were ready, they were randomly divided into 4 groups: 1) normal control; 2) HBO 1 h + 37°C incubator 2 hours; 3) LPS 3 hours in 37°C incubator; and 4) LPS 3 hours, with the first hour in HBO chamber and another 2 hours in 37°C incubator. After the treatments, the cells were collected for real-time polymerase chain reaction (RT-PCR).

Immunohistochemistry

Animals were terminally anesthetized with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. After the perfusion, the spinal cord segments (L_{3-5}) were removed and postfixed in the same fixative overnight, then replaced with 15% sucrose overnight. Spinal sections (transverse, free-floating, 30 μm) were cut in a cryostat and blocked with 2% goat serum in .3% Triton for 1 hour at room temperature and incubated overnight at 4°C with ionized calcium binding adaptor molecule 1 (IBA-1) or glial fibrillary acidic protein (GFAP) primary antibodies (anti-IBA-1, 1:1000, rabbit; Wako, Tokyo, Japan and anti-GFAP, 1:5000, mouse; Millipore, Billerica, MA). The sections were then incubated for 1 hour at room temperature with Cy3- or FITC-conjugated secondary antibodies (1:400; Jackson ImmunoResearch, West Grove, PA). The stained sections were examined with a Leica fluorescence microscope (Leica, Wetzlar, Germany), and images were captured with a CCD spot camera (Leica).

Western Blots

Animals were rapidly killed after deep anesthesia with isoflurane. The L_{3-5} spinal segments were quickly removed and homogenized in a sodium dodecyl sulfate (SDS) sample buffer containing a mixture of proteinase and phosphatase inhibitors (Sigma-Aldrich). Protein samples (25 μg) were separated on SDS–PAGE gel and transferred to nitrocellulose blots. The blots were blocked with 5% milk and incubated overnight at 4°C with antibody against phosphorylated JNK (pJNK, rabbit, 1:500; Cell Signaling, Beverly, MA), phosphorylated ERK (pERK, rabbit, 1:500; Cell Signaling), phosphorylated p38 (p-p38, rabbit, 1:500; Cell Signaling), and β-actin (mouse, 1:5000; Sigma-Aldrich). These blots were further incubated with horseradish peroxidase-conjugated secondary antibody, developed in enhanced chemiluminescence solution, and exposed onto Hyperfilm (Millipore) for 1 to 10 minutes. Specific bands were evaluated by apparent molecular size. The intensity of the selected bands was captured and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Real Time-PCR

Animals were rapidly killed after deep anesthesia with isoflurane. The L_{3-5} spinal segments were homogenized in Trizol reagent (Invitrogen). The paw skins were removed, minced into small pieces, and homogenized in Trizol. The total RNA was extracted and an OD260/280 range of 1.8 to 2.1 judged acceptable. Single-stranded cDNA was synthesized using PrimeScript RT reagent Kit (Takara Bio Inc, Otsu, Shiga, Japan) by a reverse transcript system (Eppendorf Mastercycler Progradient PCR; Eppendorf, Hamburg, Germany). Quantitative RT-PCR was performed in the Real-Time Detection System (Rotor-Gene 3000; Corbett Life Science, Hamburg, Germany) by SYBR green I dye detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression abundance was used as inner parameters to rectify the target gene expression quantity. All primers were designed via Primer Premier 5.0 (Palo Alto, CA) and were tested using an NCBI primer design tool to ensure that every pair primer was unique

Table 1. Summary of the RT-PCR Primers Sequences

Gene	Forward Primer Sequences (5′ → 3′)	Reverse Primer Sequences (5′ → 3′)
TNF-α	GTT CTA TGG CCC AGA CCC TCA C	GGC ACC ACT AGT TGG TTG TCT TTG
IL-1β	TCC AGG ATG AGG ACA TGA GCA C	GAA CGT CAC CCA GCA GGT TA
IL-6	CCA CTT CAC AAG TCG GAG GCT TA	CCA GTT TGG TAG CAT CCA TCA TTT C
MCP-1	GCA TCC ACG TGT TGG CTC A	CTC CAG CCT ACT CAT TGG GAT CA
KC	GCT TGA AGG TGT TGC CCT CAG	AGA AGC CAG CGT TCA CCA GAC
IP-10	TGA ATC CGG AAT CTA AGA CCA TCA A	AGG ACT AGC CAT CCA CTG GGT AAA G
GAPDH	AAA TGG TGA AGG TCG GTG TGA AC	CAA CAA TCT CCA CTT TGC CAC TG

to the target gene. The detailed primer sequences for each gene are listed in Table 1. The PCR reaction process was first incubated at 95°C for 30 seconds, followed by 45 cycles of thermal cycling at 95°C for 5 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. The reaction process was monitored using a Rotor-Gene Analysis Software 6.1 system (Corbett Life Science). Melt curves were performed upon completion of the cycles to ensure that nonspecific products were absent. Quantification was performed by normalizing cycle threshold (Ct) values with GAPDH Ct and analyzed with the $2^{-\Delta\Delta CT}$ method.

Quantification and Statistics

For behavioral studies, the data were analyzed with 2-way analysis of variance followed by a Bonferroni's test for post hoc analysis. For the analysis of GFAP- or IBA-1-immunoreactivity, the images of the dorsal horn were captured and a numerical value of the intensity was calculated with a computer-assisted imaging analysis system (ImageJ). The intensity of the background was subtracted in each section. The data from 3 animals in each group was averaged and analyzed. For the quantification of Western blot, the density of specific bands for pJNK, pERK, p-p38, IBA-1, GFAP, and β-actin was measured with imaging analysis software (ImageJ). The size of rectangle was fixed for each band and the background near that band was subtracted. pJNK, pERK, p-p38, IBA-1, and GFAP levels were first normalized to β-actin and then normalized to naïve. The quantification of RT-PCR was normalized to GAPDH. The immunostaining, Western blot, and RT-PCR data were analyzed with Student's t-test. All the data were presented as mean ± SEM, and $P < .05$ was considered statistically significant in all cases.

Results

HBO Treatment Reduces CFA-Induced Paw Edema and Heat Hyperalgesia

Unilateral injection of 20 μL CFA into a hindpaw of mice produced marked inflammation (Fig 1A). The volume of the inflamed paw increased at 1 hour after CFA injection, peaked at 4 days, and gradually recovered at 14 days. HBO treatment (2 ATA, pure oxygen, starting at 1 hour after CFA injection, once a day for 7 consecutive days) reduced paw volume from 3 days after CFA injection ($P < .001$, compared to control). Although the HBO treatment terminated at 6 days, the effect was maintained for more than 14 days. Treatment with pure oxygen had mild effect on the paw inflammation at some time points, whereas treatment with HBA (2 ATA, air) did not change the paw volume compared to control ($P > .05$). Additionally, HBO treatment significantly reduced severity and shortened duration of the CFA-induced heat hyperalgesia (Fig 1B). The antinociceptive effect was shown on day 2 and the duration of heat hyperalgesia was shortened from 14 to 7 days. Treatment with HBA or pure oxygen alone, in the same protocol, did not affect the pain hypersensitivity. Additionally, HBO treatment decreased the responses to .008 g and .02 g von Frey filaments from day 1 to day 14 (Figs 1C and 1D). These results indicate that HBO therapy (a combination of pure oxygen and high pressure), applied at the start of the peripheral inflammation, can effectively attenuate paw edema and pain hypersensitivity.

We then examined the possible anti-inflammatory and antinociceptive effects of HBO on ongoing inflammatory pain. Because HBA or pure oxygen did not show efficacy on the prevention of the pain behavior, here we only

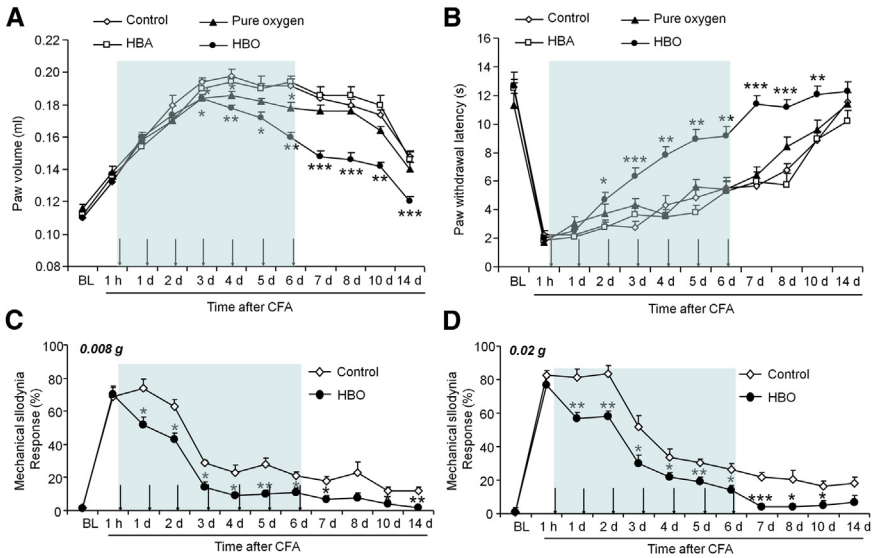

Figure 1. HBO treatment attenuates CFA-induced paw edema, heat hyperalgesia, and mechanical allodynia. Mice were treated with HBO daily on the first 7 days after intraplantar injection of CFA. HBO treatment reduces the paw volume from the third day and maintained it for more than 14 days. Pure oxygen treatment also shows mild effect on the reduction of paw volume at some time points **(A)**. HBO increases the paw withdrawal latency from the second day and maintained it for more than 14 days **(B)**. n = 5. HBO attenuates mechanical allodynia compared to the control group from the first day and maintained it for more than 14 days (tests with von Frey filaments .008 g and .02 g (**C**, **D**). *$P < .05$, **$P < .01$, ***$P < .001$, compared to control. n = 6.

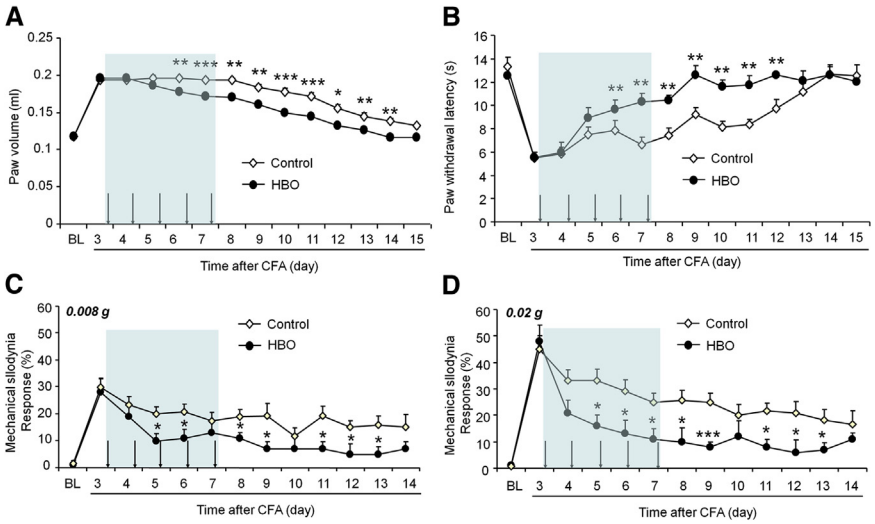

Figure 2. HBO treatment alleviates paw edema and reverses inflammatory pain. Mice were treated with HBO daily from 3 days for 5 consecutive days. HBO reduces paw edema **(A)** and heat hyperalgesia **(B)** from 6 days after CFA injection. HBO also attenuates mechanical allodynia from 5 days after CFA injection **(C, D)**. *$P < .05$, **$P < .01$, ***$P < .001$, compared to control. n = 5.

tested the effect of HBO treatment. At 3 days after CFA injection, mice were treated with HBO daily for 5 consecutive days (from day 3 to day 7). The paw volume and paw withdrawal latency were measured at 3 hours after treatment with HBO. As shown in Fig 2A, paw volume decreased at 6 days after CFA injection (3 days after HBO treatment) and was maintained until 13 days. The heat hyperalgesia was relieved at 6 days (3 days after HBO treatment) and shortened the recovery from 13 days to 9 days (Fig 2B). The mechanical allodynia was relieved from 5 days and maintained for 13 days (Figs 2C and 2D). These results demonstrate that repetitive HBO treatment is also effective in treating well-developed inflammatory pain.

HBO Treatment Suppresses CFA-Induced Activation of Astrocytes and Microglia

To investigate whether the effect of HBO on CFA-induced inflammatory pain is mediated through glial activation, we first checked the expression of astrocytic marker, GFAP, and microglial marker, IBA-1,

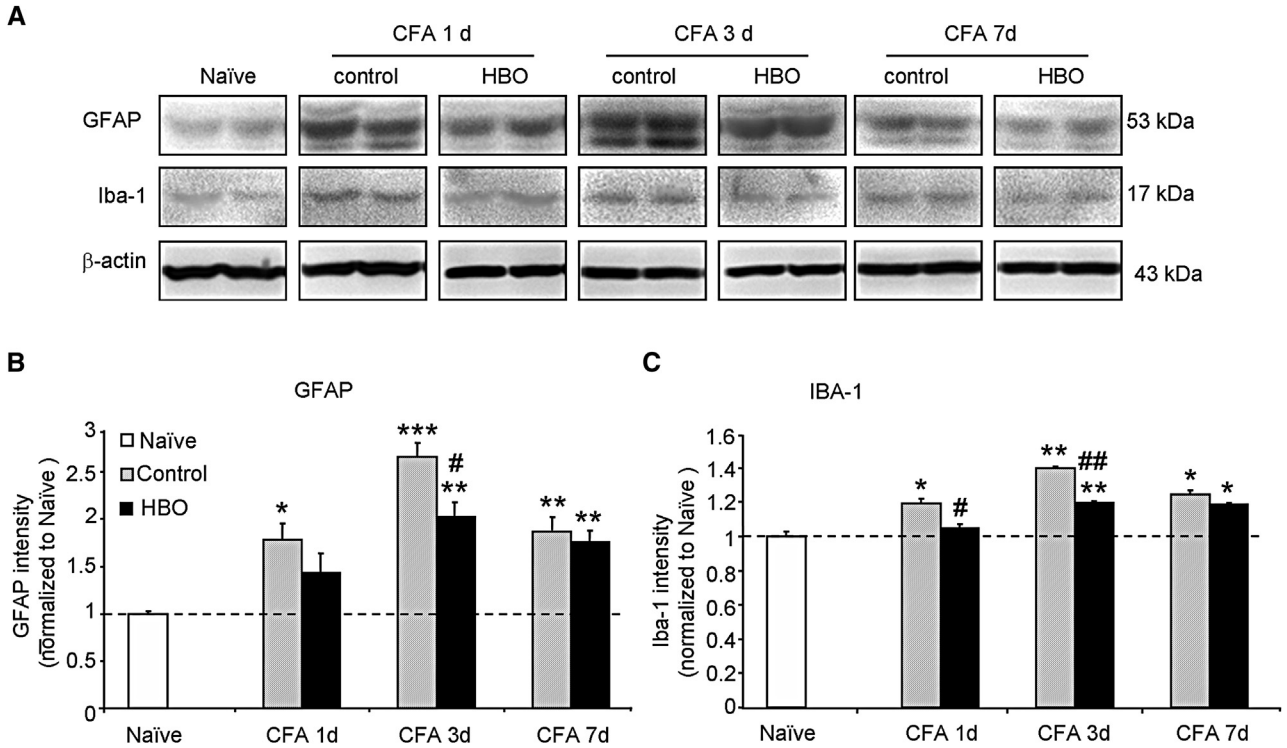

Figure 3. HBO treatment decreases CFA-induced glial activation. Western blot analysis shows that HBO treatment decreases CFA-induced upregulation of GFAP **(A, B)** and IBA-1 **(A, C)** in the spinal cord. *$P < .05$, **$P < .01$, ***$P < .001$, compared to naïve mice. #$P < .05$, ##$P < .01$, compared to control mice. n = 3.

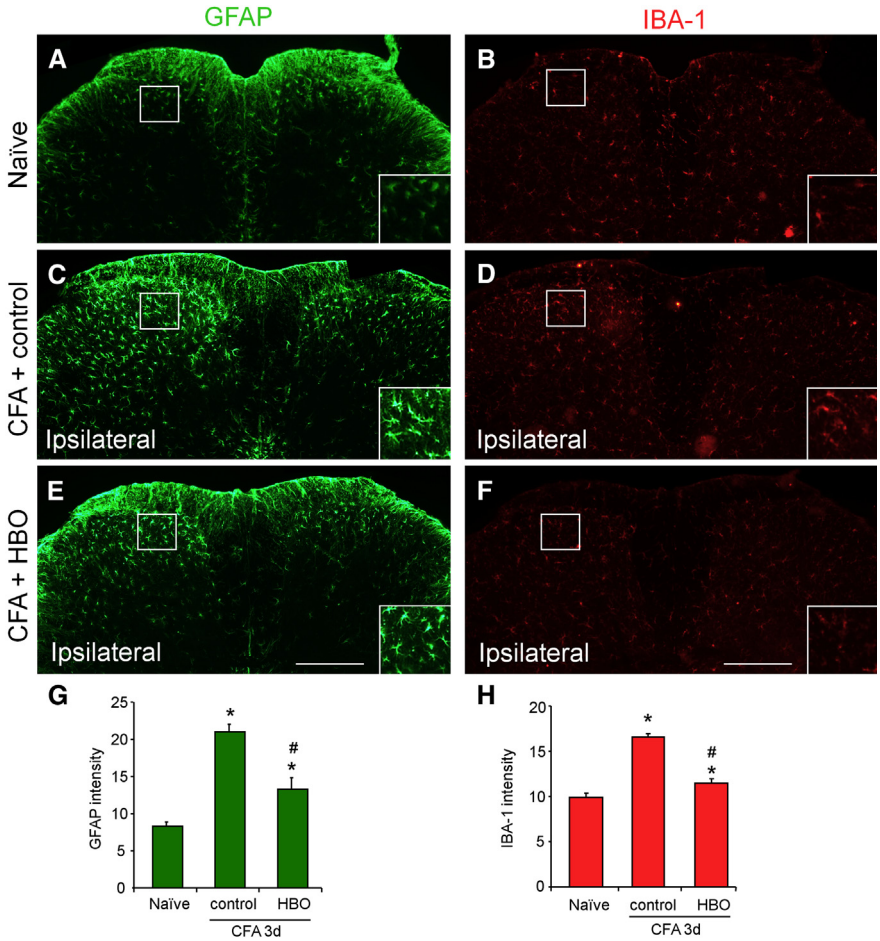

Figure 4. GFAP and IBA-1 immunostaining in the spinal cord dorsal horn of naïve mice **(A, B)** and mice that received intraplantar CFA injection as control **(C, D)** or treated with HBO **(E, F)**. The densities of GFAP-labeled astrocytes **(G)** and IBA-1-labeled microglia **(H)** in the spinal cord are compared among different groups. Mice were sacrificed 3 hours after HBO treatment, and the control group was sacrificed at the same time. Scale bar, 100 μm. *$P < .05$ compared to naïve mice. #$P < .05$ compared to control mice. n = 5.

in the spinal cord at different times after CFA injection combined with or without HBO treatment. As shown in Fig 3A, after CFA injection, the expressions of GFAP and IBA-1 were increased at 1, 3, and 7 days. HBO treatment markedly decreased GFAP expression at 3 days (Fig 3B) and IBA-1 expression at 1 and 3 days (Fig 3C).

To further check the morphological changes of astrocytes and microglia, we examined GFAP and IBA-1 expression in the spinal cord by immunofluorescence staining at 3 days after CFA injection. The HBO treatment was also given for 3 consecutive days. In naïve animals, a few GFAP-positive astrocytes and IBA-1-positive microglia were expressed (Figs 4A and 4B). At 3 days after CFA injection, a large number of GFAP-positive astroglial cells exhibited intense immunoreactivity and appeared hypertrophied with thick processes (Fig 4C). Similarly, the intensity of IBA-1-immunoreactive was also increased and the microglial processes were shortened and thickened (Fig 4D). However, after 3 days treatment with HBO, the CFA-induced activation of astrocytes and microglia was markedly reduced in spinal cord dorsal horn (Figs 4E and 4F). The intensity of GFAP staining decreased from 20.93 ± 1.08 (control) to 13.31 ± 1.58 (HBO) (Fig 4G). Similar to astrocytes, the intensity of IBA-1-positive microglia decreased from 16.56 ± .66 (control) to 11.52 ± .65 (HBO) (Fig 4H). These results suggest that CFA-induced inflammatory pain is associated with astrocytes and microglia activation[36] and that repetitive HBO treatment attenuates glial activation in mice.

HBO Treatment Suppresses CFA-Induced Activation of MAPKs in the Spinal Cord

MAPKs are important cellular signaling components, which mainly include 3 members: JNK (c-jun N-terminal kinase), ERK (extracellular signal-regulated kinase), and p38. To examine whether HBO can attenuate CFA-induced activation of MAPKs, we carried out Western blot to check protein expression of pJNK, pERK, and p-p38 in the spinal cord at 1, 3, and 7 days after CFA injection combined with or without HBO treatment. As shown in Fig 5A, CFA induced pJNK expression at 1 day, 3 days, and 7 days, which is consistent with our previous data.[14] HBO treatment did not change pJNK expression at 1 day, but significantly reduced pJNK expression at 3 and 7 days (Fig 5B). In addition, CFA increased pERK expression

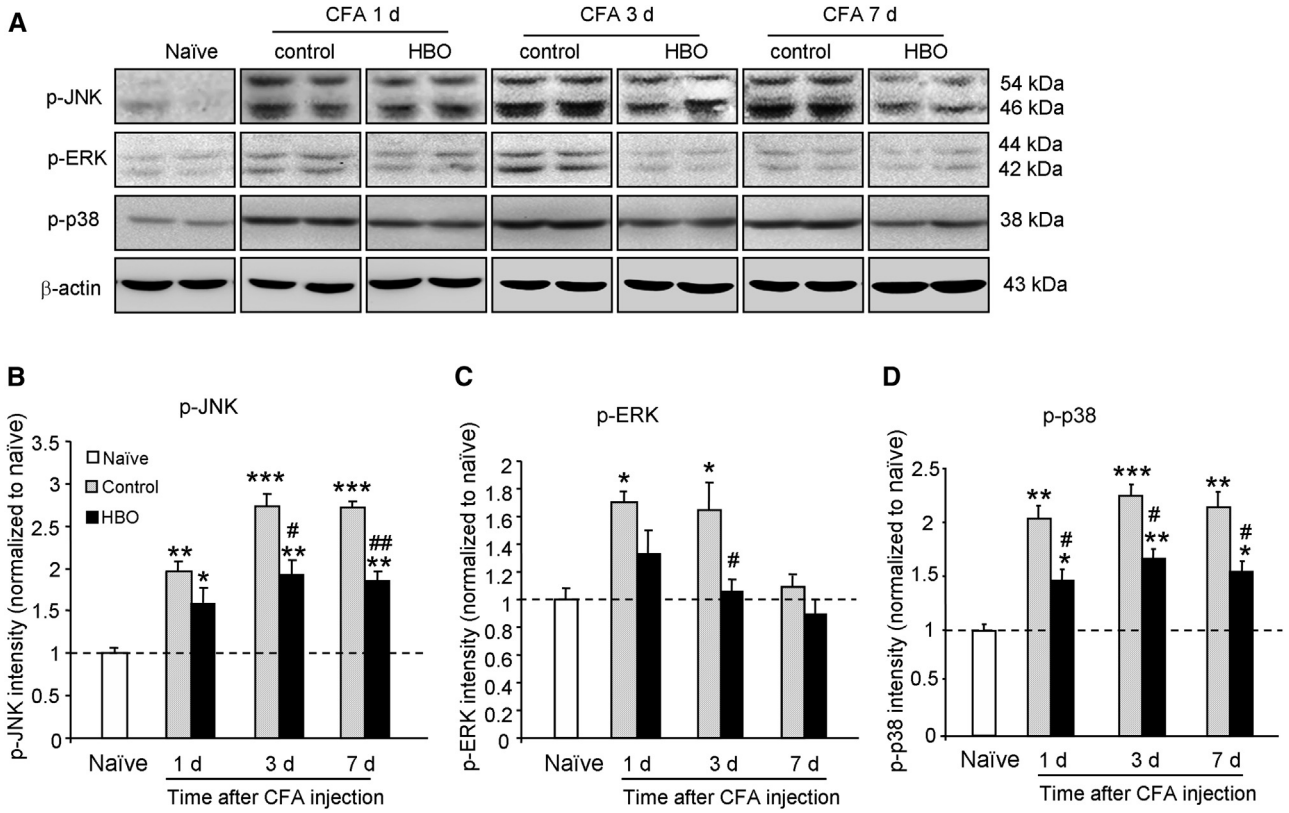

Figure 5. Western blot analysis shows the effect of HBO treatment on CFA-induced expression of pJNK, pERK, and p-p38 protein expression in the spinal cord of mice. Repetitive HBO treatment reduces CFA-induced increased pJNK, pERK, and p-p38 in ipsilateral spinal cord **(A)**. Low panels show densities of pJNK **(B)**, pERK **(C)**, and p-p38 **(D)** bands after being normalized to naïve. **P* < .05, ***P* < .01, ****P* < .001, compared to naïve mice. #*P* < .05, ##*P* < .01, compared to control mice. n = 5.

at 1 and 3 days, and HBO treatment blocked CFA-induced pERK upregulation at 3 days (Fig 5C). HBO treatment also markedly reduced p-p38 expression at 1, 3, and 7 days (Fig 5D).

HBO Treatment Reduces CFA-Induced Upregulation of Proinflammatory Cytokines and Chemokines in the Spinal Cord and Primary Cultured Astrocytes and Microglia

It was known that glial cells release a variety of mediators including proinflammatory cytokines and chemokines that contribute to chronic pain.[15,49] Therefore, we checked the mRNA expression in the spinal cord after CFA and HBO treatment (Fig 6A). In naïve animals, proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and chemokines MCP-1/CCL2, KC/CXCL1, and IP-10/CXCL10, had low expression in the spinal cord. At 3 days after CFA injection, the mRNA of TNF-α, IL-1β, MCP-1, KC, and IP-10 were significantly increased, with IL-1β and IP-10 increased more than 5-fold. Importantly, HBO treatment effectively decreased CFA-induced mRNA upregulation of TNF-α, IL-1β, MCP-1, and IP-10. However, the mRNA expression of KC and IL-6 was not affected by HBO.

To further check if these mediators are expressed in glial cells and the effect of HBO on their expression, we primarily cultured astrocytes and microglia separately. To mimic the neuroinflammation in vitro, we used LPS (1 μg/mL) to incubate with glial cells for 3 hours. As shown in Fig 6B, the cytokines and chemokines that we checked were constitutively expressed in astrocytes. LPS incubation for 3 hours dramatically increased their expressions, with the increase of TNF-α and KC mRNA by more than 120-fold, and IL-1β, MCP-1, and IP-10 mRNA more than 40-fold. HBO treatment alone did not show significant effect on the mRNA expression of these cytokines and chemokines. However, treatment with HBO for 1 hour during LPS incubation significantly reduced LPS-induced mRNA upregulation of these cytokines and chemokines. In cultured microglia, LPS dramatically increased TNF-α expression more than 140-fold. LPS also significantly increased the mRNA expression of IL-1β, IL-6, MCP-1, KC, and IP-10. HBO treatment decreased their upregulation at mRNA level (Fig 6C).

HBO Treatment Reduces CFA-Induced Upregulation of Proinflammatory Cytokines and Chemokines in the Inflamed Paw

Because HBO treatment decreased the edema of the inflamed paw, we checked if HBO could inhibit the expression of inflammatory mediators in the paw. At 3 days after CFA injection, IL-1β, IL-6, MCP-1, and IP-10 were dramatically increased (Fig 7). Particularly, IL-6 mRNA was increased 700-fold. HBO treatment

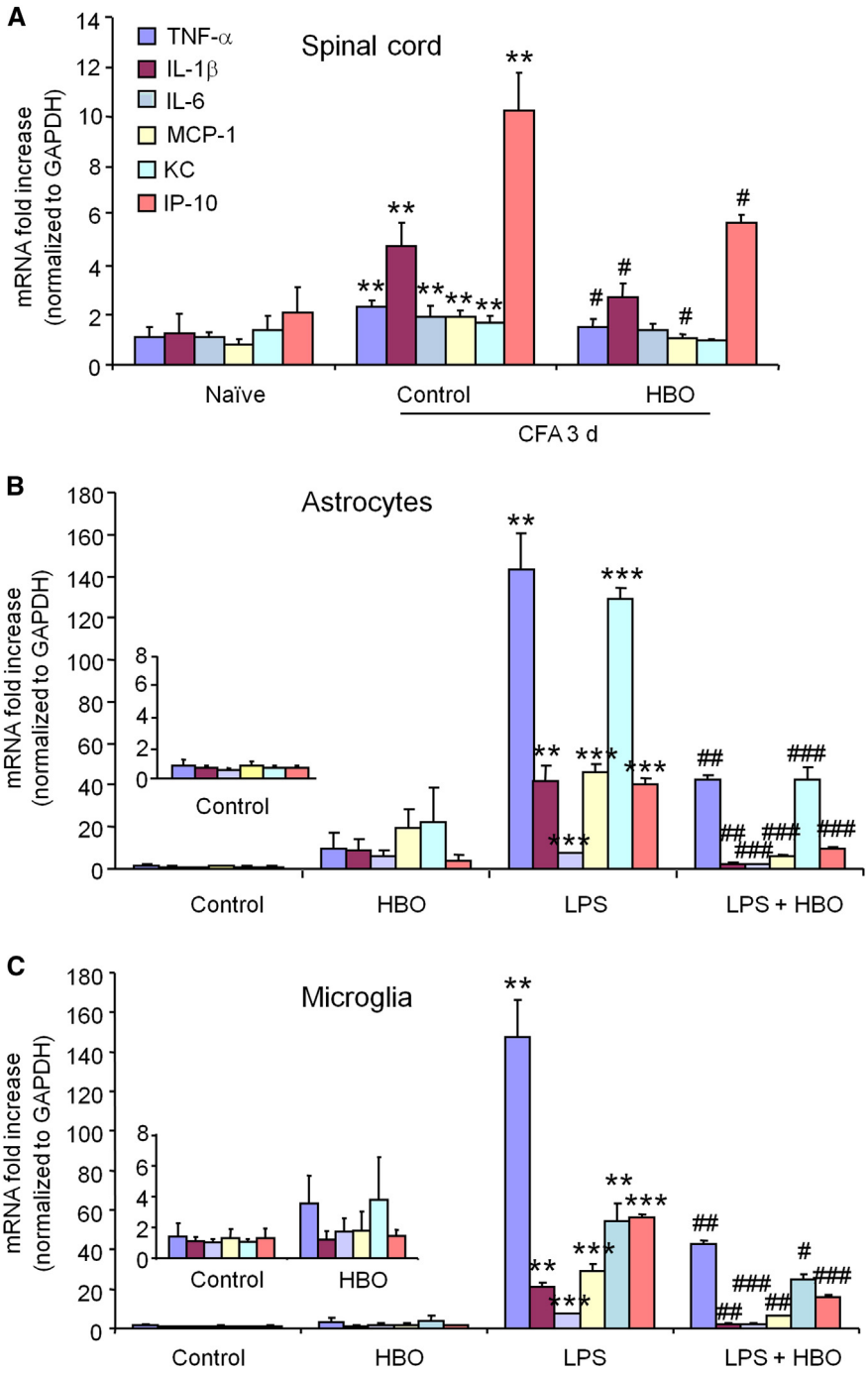

Figure 6. HBO treatment decreases the expression of proinflammatory cytokines and chemokines expression in the spinal cord, primary cultured astrocytes, and microglia. **(A)** CFA increases the mRNA expression of TNF-α, IL-1β, IL-6, MCP-1, KC, and IP-10. HBO treatment decreases the expression of TNF-α, IL-1β, MCP-1, and IP-10 in the spinal cord **(A)**. LPS increases the mRNA expression of TNF-α, IL-1β, IL-6, MCP-1, KC, and IP-10 in astrocytes **(B)** and microglia **(C)**, which is decreased by HBO treatment. **P* < .05. ***P* < .01, ****P* < .001, compared to control. #*P* < .05, ##*P* < .01, ###*P* < .001 compared to LPS treatment. n = 5.

significantly decreased the mRNA expression of IL-1β, IL-6, MCP-1, KC, and IP-10. However, in contrast with significant changes of TNF-α in the spinal cord and cultured cells, HBO had no effect on TNF-α expression in the paw skin (Fig 7).

Discussion

In this study, we investigated the antinociceptive effect of repetitive treatment with HBO on CFA-induced inflammatory pain and explored the possible mechanisms. Our results demonstrated first that HBO repetitive treatment in both early and later phases produced a prolonged antinociceptive and anti-inflammatory effect in animals that persisted after cessation of the treatment. Second, HBO treatment decreased the spinal activation of glial cells (astrocytes and microglia) and phosphorylation of MAPKs (JNK, ERK, p38) induced by CFA. Third, HBO reduced

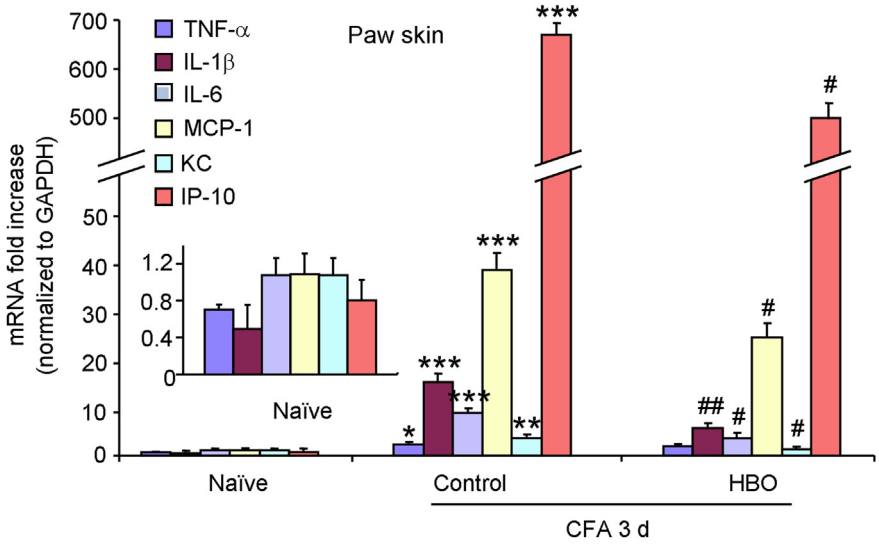

Figure 7. HBO treatment decreases the expression of proinflammatory cytokines and chemokines in the inflamed paw skin. CFA increases the mRNA expression of TNF-α, IL-1β, IL-6, MCP-1, KC, and IP-10. HBO treatment decreases the CFA-induced upregulation of IL-1β, IL-6, MCP-1, KC, and IP-10 mRNA. *P < .05, **P < .01, ***P < .001, compared to naïve. #P < .05, ##P < .01, compared to CFA control. n = 5.

CFA-induced production of several proinflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (MCP-1, KC, IP-10) in the spinal cord and the inflamed paw and LPS-induced inflammatory mediators' production in cultured astrocytes and microglia in a varied pattern. These data suggest that HBO therapy might be an effective approach for alleviating chronic inflammatory pain through, at least partially, suppression of the inflammation in both spinal cord and the inflamed paw.

Repetitive HBO Attenuates Chronic Inflammatory Pain and Paw Edema

HBO therapy has been clinically used for the protection of the central nervous system after acute injury.[31,41] In recent years, HBO therapy is reported to be effective in treating several clinical pain conditions in patients.[18,53] Animal studies show that HBO attenuates neuropathic pain induced by spinal nerve ligation or CCI of the sciatic nerve.[18,28,44] In addition, it was shown that HBO decreases peripheral injection of carrageenan-induced paw edema[43,51] and acute pain.[51] HBO also alleviates joint inflammation and reduces mechanical hyperalgesia in an animal model of arthritis, and the effect is comparable to acetylsalicylic acid.[50] Our results first demonstrated that repetitive HBO treatment decreased heat hyperalgesia, mechanical allodynia, and paw edema associated with chronic inflammatory pain conditions. It should be noted that the anti-inflammatory and antinociceptive effect of HBO on chronic inflammatory pain could only be obtained by repetitive, not single, treatment of HBO. In addition, paw edema was comparable between the HBO and control groups in the first 2 days and started to show a decrease at 3 days, whereas the antinociceptive effects are apparent at 1 day after CFA injection, suggesting a dissociation of the paw edema with pain hypersensitivity. This is consistent with the results obtained by Wilson et al[51] in a carrageenan-induced acute inflammatory pain model, further supporting that distinct mechanisms might be involved in the anti-inflammatory and antinociceptive properties of HBO therapy. Our results also showed that HBO produced a prolonged antinociceptive and anti-inflammatory effect that persisted after cessation of treatment, indicating that HBO may be involved in the modulation of some mechanisms that contribute to the maintenance of chronic pain.

Repetitive HBO Treatment Reduces Glial Activation and Inflammatory Mediators' Production

In recent years, non-neuronal cells such as immune cells and glial cells have been implicated to play a critical role in the pathogenesis of chronic pain.[5,30,34,38,47] Both astrocytes and microglia are activated in the spinal cord following peripheral nerve injuries or tissue injury, and the activated glial cells can contribute to the enhancement and maintenance of chronic pain by activating intracellular signals (eg, MAPKs) and releasing neuromodulators, such as growth factors, proinflammatory cytokines, and chemokines.[1,20,33,46] Behavioral studies show that blocking the activation of spinal cord microglia with minocycline[27,32,35] and astrocytes with fluorocitrate and L-a-AA[13,56] prevents or delays the development of pain hypersensitivity. Therefore, we checked whether HBO could regulate the activity of glial cells in the spinal cord. Our data showed that HBO treatment decreased CFA-induced GFAP upregulation at 3 days and IBA-1 upregulation at 1 and 3 days. In agreement with our results, Gu et al[18] recently showed that HBO treatment decreased

CCI-induced GFAP upregulation in the spinal cord. These data suggest that HBO may be involved in the regulation of glial functions.

The MAPKs JNK, ERK, and p38 are important molecules in chronic pain sensitization and are differentially activated in spinal cord glial cells.[22] Inhibition of MAPKs by inhibitors of ERK, JNK, or p38 has shown antinociceptive effect on neuropathic pain.[22] Here we showed that CFA induced upregulation of pJNK, pERK and p-p38. HBO treatment decreased pJNK expression at 3 and 7 days, pERK expression at 3 days, and p-p38 expression at all 3 time points. It was reported that repetitive treatment suppresses CCI-induced pERK expression in the spinal cord.[18] These results suggest that the inhibition of MAPKs is associated with the antinociceptive effect of HBO.

Glial cells express and release a variety of proinflammatory cytokines and chemokines. Our previous results showed that the protein expression of MCP-1, KC, and IP-10 were regulated by JNK pathway in astrocytes.[16] Here we first showed that CFA induced significant mRNA upregulation of TNF-α, IL-1β, IL-6, MCP-1, KC, and IP-10 at 3 days after CFA injection. In vitro results supported the expression of these mediators by astrocytes and microglia. HBO treatment markedly inhibited the mRNA upregulation of proinflammatory cytokines and chemokines both in vivo and in vitro. However, it is noteworthy that the expression levels of these mediators and inhibition levels by HBO are different among spinal cord, paw skin, and cell cultures, which may be partially due to the differential basal expression of these mediators in different tissues.[16] On the other hand, HBO may have different effects on intracellular signaling in different cells, which needs to be further investigated. In addition, as the activity of these factors is mediated by their protein, whether the protein levels of these mediators have similar changes also needs to be confirmed in the future. Electrophysiological results indicate that TNF-α and MCP-1 enhance excitatory synaptic transmission, IL-6 decreases inhibitory synaptic transmission, and IL-1β both enhances excitatory synaptic transmission and decreases inhibitory synaptic transmission in dorsal horn neurons,[16,24] suggesting that they directly regulate neuronal excitability. Gu et al[18] also showed that HBO suppressed CCI-induced phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B), calmodulin-dependent kinase II (CaMKII), and cAMP response element-binding protein (CREB) in the spinal cord at 14 postoperative days, suggesting that HBO can effectively reverse the increased neural activities. Therefore, the decreased activation of glial cells and reduced expression of inflammatory cytokines and chemokines in the spinal cord may contribute to the antinociceptive effect of HBO through decreased neuronal excitability. Besides, HBO may induce nitric oxide-dependent release of opioid peptide to cause a long-acting antinociceptive effect.[6,54]

The Peripheral Anti-inflammation Effect of Repetitive HBO Treatment

Although central sensitization in the spinal cord level plays an important role in the development and maintenance of chronic pain, the peripheral mechanism is also involved. Following tissue injury, an inflammatory response is generated by local macrophages and this is further amplifled by migrating blood cells. The various inflammatory mediators act synergistically to induce and maintain the development of pain hypersensitivity.[42] Behavioral studies show that intraplantar injection of TNF, IL-1β, IL-6, or KC differentially induces pain.[7,8,10,29,45] Intraplantar injection carrageenan induces the expression of TNF-α, IL-1β, IL-6, MCP-1, and MIP-1α.[52] Cytokine antagonists are further able to reduce carrageenan-induced hyperalgesia.[45] These data indicate that peripheral activation of cytokines and chemokines is an important step in the development of inflammatory pain. Here, we show that CFA injection increased the mRNA expression of TNF-α, IL-1β, IL-6, MCP-1, KC, and IP-10 at 3 days. HBO treatment decreases their expression in the paw skin with reduced paw edema. Li et al[28] reported that HBO reduces CCI-induced TNF-α upregulation in the sciatic nerve. These data suggest that the peripheral mechanism is also involved in the antinociceptive effect of HBO therapy.

In conclusion, this study demonstrated that HBO treatment is an effective approach to relieve CFA-induced chronic pain and paw edema. This effect may be mediated by inhibition of glia-mediated neuroinflammation in the spinal cord and the production of inflammatory mediators in the peripheral tissue.

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