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Hyperbaric Oxygen Preconditioning Attenuates Hemorrhagic Transformation through Increasing PPAR γ in Hyperglycemic MCAO Rats

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Abstract

Hyperbaric oxygen preconditioning (HBO-PC) has been demonstrated to attenuate hemorrhagic transformation (HT) after middle cerebral artery occlusion (MCAO) in hyperglycemic rats. However, the mechanisms remain to be illustrated. Recently, HBO-PC has been shown to activate peroxisome proliferator-activated receptor-gamma (PPAR γ) by increasing 15d-PGJ2 in primary cultured neurons. We hypothesize that HBO-PC reduces HT by suppressing inflammation through increasing 15d-PGJ2 and activating PPAR γ in hyperglycemic MCAO rats. HBO (2.5 ATA) was administered for 1 hour daily for 5 consecutive days. The PPAR γ inhibitor GW9662 was administered intraperitoneally to designated animals. Infarction volume, hemorrhage volume, neurological scores and mortality were analyzed. The levels of 15d-PGJ2, PPAR γ , TNF- α and IL-1 β , tight junction proteins as well as the activity of MMP-2 and MMP-9 were evaluated 24 hours after MCAO. HBO-PC reduced HT, improved neurological function, down-regulated inflammatory molecules and inhibited the activation of MMP-9 by increasing 15d-PGJ2 and PPAR γ at 24 hours after MCAO. The results suggested HBO-PC might be an alternative measure to decrease HT in ischemic stroke.

Keywords

Hyperbaric oxygen preconditioning; PPAR γ ; hemorrhagic transformation; MCAO

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Introduction

Hemorrhagic transformation (HT) is one of the major side effects of tissue plasminogen activator (tPA) treatment and occurs in 5% to 10% of stroke patients (Donnan et al., 2011). Emerging data show that pre-ischemic hyperglycemia dramatically aggravated brain infarct and HT in middle cerebral artery occlusion (MCAO) rat model (Cipolla and Godfrey, 2010; Paciaroni et al., 2009; Xing et al., 2011). The development of HT in hyperglycemic rats may be associated with increased oxidative stress and inflammatory activity (Fabian and Kent, 2013), which causes blood brain barrier (BBB) disruption (Chiu et al., 2013; Paciaroni et al., 2009; Fagan et al., 2013). Preventing inflammation and stabilizing BBB against breakdown may reduce tPA side effects and improve patient outcomes (Hafez et al., 2014).

Preconditioning has been reported to be a promising therapeutic strategy against the ischemia/reperfusion injury in experiment animals (Dezfulian et al., 2013; Koch, 2012). Previously we have demonstrated that hyperbaric oxygen preconditioning (HBO-PC) strongly suppressed HT and improved neurological deficits in hyperglycemic MCAO rats (Soejima et al., 2013; Soejima et al., 2012); however, the mechanisms remain to be illustrated. Peroxisome proliferator activated receptor- γ (PPAR γ) is a member of the nuclear hormone receptor superfamily, and plays a central role in the regulation of apoptosis (Chang and Szabo, 2000), oxidative stress and inflammation (Lehrke and Lazar, 2005) as well as affords significant neuroprotection against cerebral ischemia reperfusion injury (Wu et al., 2009). 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) is an endogenous ligand with a high affinity for PPAR γ . Activation of PPAR γ has been reported to preserve the integrity of the BBB in type 2 diabetic mice (Min et al., 2012). Recently, Zeng and his colleagues found that HBO-PC significantly increased the levels of 15d-PGJ2, PPAR γ mRNA and protein in primary cultured cortical neurons with OGD exposure (Zeng et al., 2012). Therefore, in the present study, we will investigate whether HBO-PC attenuates hyperglycemia-induced HT in MCAO rats and protect BBB through increasing 15d-PGJ2 and activating PPAR γ signal pathway.

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Animal Groups and Interventions

Two hundred and eighty-six male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). HBO at 2.5 ATA was administered for 1 hour daily for 5 consecutive days. 50% dextrose (DX, 6 ml/kg) was injected intraperitoneally 30 minutes before MCAO to induce acute hyperglycemia. PPAR γ inhibitor GW9662 was administered intraperitoneally to designated animals for intervention. Animals were divided into six groups: DX+sham (n=20), DX+MCAO (n=54), DX+HBO+MCAO (n=50), DX+NBO+MCAO (n=50), DX+HBO+MCAO+GW9662 (n=50), and DX+HBO+MCAO+DMSO (n=48). Infarction volume, hemorrhage volume, neurological scores and mortality were

measured for outcome study. The protein level of PPAR γ , TNF- α and IL-1 β , as well as the activity of MMP-2 and MMP-9 were evaluated at 24 hours after MCAO. Two additional groups of animals (Naïve and Naïve+HBO-PC, n=6 for each group) were added to measure the effects of HBO-PC on the level of 15d-PGJ2 in naïve rats.

Hyperglycemia Induction and MCAO

All rats received 50% dextrose (6 ml/kg) intraperitoneally 30 minutes before MCAO to induce acute hyperglycemia. Anesthesia was induced with ketamine and xylazine (80 mg/kg and 10 mg/kg respectively, intraperitoneally), followed by atropine at a dose of 0.1 mg/kg subcutaneously. During the surgery and postoperative period, rectal temperature was maintained at 37.0°C by using a feedback-controlled heating pad. MCAO was performed as previously reported (Hu et al., 2011). Briefly, the right external carotid artery was isolated and coagulated. A 4-0 nylon suture with a round tip was inserted into the internal carotid artery through the external carotid artery stump and advanced to occlude the origin of middle cerebral artery. The suture was removed at 2 hours after occlusion. Sham operated rats underwent the same surgical procedures without insertion of the suture.

HBO-PC Regimen

Due to its potential toxic effects, HBO is currently restricted to short sessions (less than 2 hours), at pressures below the threshold of central nervous system toxicity (0.3 MPa) (Tibbles and Edelsberg, 1996). In our previous studies and preliminary experiments, we tested HBO-PC at 1, 1.5, 2, 2.5, and 3 ATA and found 2.5–3 ATA produced more pronounced protective results compared to 1–2 ATA. Therefore in this study, we used HBO at 2.5 ATA for 1 hour.

Rats were pressurized in a research hyperbaric chamber (1300B; Sechrist) at 2.5 atmospheres absolutes with 100% oxygen (flow of 22 L/min). Compression and decompression were maintained at a rate of 5 psi/min. HBO was administered for 1 hour daily for 5 consecutive days and the last session was performed 24 hours before MCAO. In the intervention groups, PPAR inhibitor GW9662 (4mg/kg) or 10% dimethyl sulfoxide (DMSO, vehicle) was injected intraperitoneally 1 hour before MCAO.

2,3,5-triphenyltetrazolium Chloride (TTC) Staining

TTC staining was performed to determine the infarct volume at 24 hours after MCAO as previously reported (Hu et al., 2011). The possible interference of brain edema with infarct volume was corrected by standard methods (whole contralateral hemisphere volume – nonischemic ipsilateral hemisphere volume) and the infarcted volume was expressed as a percentage of the whole contralateral hemisphere.

Spectrophotometric Assay of Hemoglobin

Hemorrhagic volume was quantified with spectrophotometric assay of brain hemoglobin content at 24 hours after MCAO. Cerebral hemorrhage was quantified using a previously described spectrophotometric assay (Hu et al., 2011). A standard curve was obtained using a “virtual” model of hemorrhage. Incremental volumes of homologous blood (0, 2, 4, 8, 16, 32 μ l) were added to the perfused brain tissue. The hemispheric brain was then homogenized in

distilled water followed by 30-minute centrifugation (13,000 g). Drabkin reagent (1.6 ml; Sigma) was added to 0.4 ml supernatant aliquots and optical density was measured at 540 nm via spectrophotometer (Spectronix 3000; Milton-Roy). Hemoglobin measurements were performed and compared with the standard curve to obtain data in terms of hemorrhage volume. The total hemispheric hemoglobin content was expressed as μl of blood per hemisphere.

Neurological Scores

A neurological examination was performed by a blinded investigator as previously described with modifications (Garcia et al., 1995) at 24 hours after MCAO. The scores given to each rat at completion of the evaluation was the summation of 7 individual test scores (spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, and beam walking). The neurological scoring ranged from 3 (most severe deficit) to 21 (maximum).

Enzyme-linked Immunosorbent Assay (ELISA) for 15d-PGJ2

15d-PGJ2 brain levels were determined using ELISA kit (MyBioSource, Inc, Canada). Rats were fatally anesthetized with isoflurane (5%) followed by cardiovascular perfusion with ice-cold 0.01M phosphate buffered saline. Brains were flash frozen in liquid nitrogen immediately following removal and kept in -80°C . Right hemisphere of the brain tissues were homogenized by sonication in an ice-cold 0.01M phosphate buffered saline and centrifuged for 15 minutes at $1500\times g$. The supernatants were collected and Aliquots were stored at -80°C until tested. Enzyme immunoassay isolation and prostaglandin quantification were carried out following manufacturer's instructions. Protein concentration of each sample was determined using a Bio-Rad protein assay kit.

Western Blot Analysis

Animals were anesthetized and underwent transcardiac perfusion using 0.01 M PBS until colorless perfusion fluid was obtained from the right atrium. Tissue samples of the ipsilateral hemisphere were obtained and immersed in 0.5 ml of the Western blot sample buffer and then sonicated for Western blot analysis. Protein concentration of each sample was determined using a Bio-Rad protein assay kit. The Western blot analysis was performed as previously described (Hu et al., 2011). Primary antibodies used were: PPAR γ (sc-271392, Santa Cruz Biotechnology), TNF- α (sc-243, Santa Cruz Biotechnology), IL-1 β (sc-554859, Santa Cruz Biotechnology), ZO-1 (sc-10804, Santa Cruz Biotechnology), Occludin (sc-5562, Santa Cruz Biotechnology) and β -actin (sc-1616, Santa Cruz Biotechnology).

MMPs Zymography

Similarly prepared protein samples as for Western blot were subjected to gelatin zymography. Approximately 50 μg of each sample was loaded per lane into the well of precast gels (10% polyacrylamide minigels containing 0.1% gelatin; Invitrogen) with SDS running buffer (1:1; Novex). Electrophoresis was performed with a Tris-glycine running buffer at 125 V constant voltage for 1.5–2 h. The gel was removed and incubated for 1 hour at room temperature in 100 ml of 2.7% Triton X-100 (renature buffer, Invitrogen) on a

rotary shaker, then washed with distilled water 10 times. Each gel was incubated with 100 ml of development buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM CaCl₂, and 0.2% Brij 35; Invitrogen) at 37°C for 14 hours on a rotary shaker. Staining was performed with 100 ml of 0.5% Coomassie blue G-250 in 30% methanol and 10% acetic acid for at least 1 hour, and gels were then destained with three changes of solutions. Gelatinolytic activity was demonstrated as clear zones or bands at the appropriate molecular weights. Human MMP-9 and human MMP-2 (from Chemicon Temecula, CA) were used as standards. The activity of MMP-2 and MMP-9 was quantified in ImageJ software.

Statistical Analyses

Data were expressed as the mean $\pm$ SEM. Statistical differences among groups were analyzed by using ANOVA followed by the Tukey method. Mortality was evaluated by Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

HBO-PC attenuated HT after MCAO in hyperglycemic rats dependent on activation of PPAR γ

Hyperglycemia induced extensive HT in ischemic territories at 24 hours after MCAO (Figures 1A and 1B). HBO-PC reduced infarct volume (Figures 1A and 1B, $p < 0.05$ vs. DX +MCAO) and hemorrhage volume (Figure 1C, $p < 0.05$ vs. DX+MCAO) significantly. After MCAO, animals showed obvious neurological deficits (Figure 2A, $p < 0.05$ vs. DX+Sham) and exhibited serious brain edema (Figure 2B, $p < 0.05$ vs. DX+Sham); HBO-PC significantly improved neurological functions (Figure 2A, $p < 0.05$ vs. DX+MCAO) and attenuated brain edema (Figure 2B, $p < 0.05$ vs. DX+MCAO) at 24 hours after MCAO. Blocking PPAR γ by its inhibitor GW9662 after each HBO-PC abolished the beneficial effects of HBO-PC (Figure 1 and 2, $p < 0.05$ vs. DX+MCAO +HBO). NBO showed the tendency to ameliorate the outcomes, but no significant difference (Figure 1 and 2, $p > 0.05$ vs. DX+MCAO).

The mortality at 24 hour after surgery in each group is: Sham+DX, 0 (0/20); DX+MCAO, 0.3333 (18/54); DX+MCAO+NBO, 0.2800 (14/50); DX+MCAO+HBO, 0.2400 (12/50); DX+MCAO+HBO+vehicle, 0.2500 (12/48); DX+MCAO+HBO+GW9662, 0.3077 (16/52). HBO-PC showed the tendency to decrease mortality, but there was no significant difference compared with DX+MCAO group (Figure 2C, $p > 0.05$ vs. DX+MCAO).

The blood glucose levels at 2 hours after injection of dextrose in all groups were significantly higher than the baseline and high glucose level lasted until 6 hours after injection. HBO-PC had no effects on the blood glucose level (Figure 2D).

HBO-PC increased the level of 15d-PGJ2 and PPAR γ at 24 hour after MCAO

We measured the levels of PPAR γ ligand 15d-PGJ2 in brain tissue by ELISA and evaluated the expression of PPAR γ by Western blots. HBO-PC significantly increased the level of 15d-PGJ2 in naïve rats 24 hours after the last HBO exposure. (Figure 3A, $p < 0.05$ vs. Naïve). The level of 15d-PGJ2 in sham and MCAO animals were relatively low, and HBO-PC

significantly increased the level of 15d-PGJ2 at 24 hours after MCAO (Figure 3B, $p<0.05$ vs. DX+MCAO). PPAR γ was seldom expressed in sham animals, and mildly increased 24 hours after MCAO (Figure 3C, $p>0.05$ vs. DX+Sham). HBO-PC greatly up-regulated the expression of PPAR γ (Figure 3C, $p<0.05$ vs. DX+MCAO).

HBO-PC suppressed inflammation and attenuated BBB disruption by decreasing MMP-9 at 24 hour after MCAO

Twenty-four hours after MCAO, the expression of inflammatory molecules TNF- α and IL-1 β increased, and HBO-PC effectively suppressed the increase of TNF- α and IL-1 β (Figure 4, $p<0.05$ vs. DX+Sham). Administration of GW9662 before each HBO-PC removed the effects of HBO-PC (Figure 4, $p<0.05$ vs. DX+MCAO+HBO-PC). The protein level of tight junction proteins ZO-1 and Occludin dramatically decreased after MCAO, and increased by HBO-PC (Figure 4, $p<0.05$ vs. DX+MCAO).

In hyperglycemic rats, the activity of MMP-9 was greatly increased at 24 hours after MCAO ($p<0.05$ vs. DX+Sham, Figure 5A, 5B), HBO-PC remarkably decreased the activity of MMP-9 ($p<0.05$ vs. DX+MCAO, Figure 5A, 5B); PPAR γ inhibitor GW9662 reversed the results of HBO-PC and increased the activity of MMP-9 ($p<0.05$ vs. DX+MCAO+HBO-PC, Figure 5A, 5B). HBO-PC had no remarkable effect on the activity of MMP-2 ($p>0.05$ vs. DX+MCAO, Figure 5A, 5C).

Discussion

In the present study, we observed that HBO-PC ameliorated the hyperglycemia-enhanced HT and improved neurologic deficits in rats subjected to MCAO. Administration of PPAR γ inhibitor GW9662 abolished the beneficial effects of HBO-PC. HBO-PC prevented HT by down-regulation of inflammatory molecules TNF- α and IL-1 β and suppressing the activity of MMP-9, which are mediated by up-regulating 15d-PGJ2 and activating PPAR γ . Our results strongly suggested that HBO-PC might turn out to be an attractive strategy for HT in ischemic stroke patients.

15d-PGJ2 is one of the prostaglandins of the J series (PGJs), which are cyclopentenones synthesized from arachidonic acid via enzymatic conversion by cyclooxygenases (COXs, COX-1 and COX-2, respectively) and PGD synthase followed by non-enzymatic dehydration from PGD2 to a series of PGJs (Nosjean and Boutin, 2002). Acting as a physiologic agonist for PPAR γ , 15d-PGJ2 has been considered as a potential endogenous protectant counteracting deleterious actions of the pro-inflammatory response in various stressful conditions. An analysis of 552 patients with an acute stroke admitted within 24 hours after symptom onset showed that increased plasma 15d-PGJ2 concentration is associated with good early and late neurologic outcome and smaller infarct volume, suggesting beneficial role of 15d-PGJ2 for stroke patients (Blanco et al., 2005). In experiment stroke models, 15d-PGJ2 has been proven to be neuroprotective through anti-oxidation (Lin et al., 2006) and anti-inflammation (Zhao et al., 2006). HBO-PC protected primary cultured cortical neurons against oxygen-glucose deprivation by releasing 15d-PGJ2 through activation of COX-2 (Zeng et al., 2012). In agreement with these studies, we observed an improvement of neurological function and attenuation of HT in hyperglycemic

MCAO rats after HBO-PC, which is associated with suppressing inflammation and preserving tight junction proteins by the increasing 15d-PGJ2. The rate-limiting step in the prostaglandin biosynthesis is the COX-catalyzed conversion of arachidonic acid mobilized from the membrane phospholipid to prostaglandin H2 (PGH2) (Surh et al., 2011). In the mouse model of surgical brain injury or rat model of transient global cerebral ischemia, HBO-PC alone significantly increased COX-2 levels (Cheng et al., 2011; Jadhav et al., 2009). Oxygen has been showed to promote prostacyclin synthesis in intact intrapulmonary arteries, which may be mediated by increasing the activity of COX-2 (Shaul et al., 1992; Shaul et al., 1993).

After activated by 15d-PGJ2, PPAR γ forms a heterodimer with another nuclear receptor PPAR-retinoid X receptor (RXR)- α and bind to PPAR response elements in the promoter of their target genes. Recent data have shown that PPAR γ acts as a regulator of CNS inflammation and is a powerful pharmacological target for CNS disease (Gillespie et al., 2011). After stroke, the activation of PPAR γ can antagonize the harmful effects of oxidative stress and inflammation, indicating a promising and neuroprotective role for PPAR γ agonists in stroke (Culman et al., 2007). Activation of PPAR γ attenuated the expression of ICAM-1, matrix metalloproteinase (MMP)-9 and various inflammatory cytokines in ischemic brain tissue (Luo et al., 2006; Pereira et al., 2005; Sundararajan et al., 2005). In agreement with these studies, HBO-PC activated PPAR γ signaling pathway and significantly decreased inflammation and suppressed the activity of MMP-9 in the present study. HBO-PC attenuated the BBB disruption and therefor reduced HT in the hyperglycemic MCAO rats.

In conclusion, we demonstrated that hyperglycemia enhanced HT after MCAO; HBO-PC attenuated HT and improved neurological function in MCAO rats. HBO-PC preserved the integrity of BBB by suppressing inflammation through activating PPAR γ pathway and decreased inflammation. Our results suggested that HBO-PC might be a promising approach to reduce HT in ischemic stroke.

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Highlights

- HBO-PC improved neurological deficits and reduced hemorrhagic transformation after MCAO.
- HBO-PC increased the level of 15d-PGJ2 and PPAR γ .
- HBO-PC decreased inflammation and suppressed the activity of MMP-9.
- PPAR γ inhibitor GW9662 removed the effects of HBO-PC.
- HBO-PC has potentials to reduce hemorrhagic transformation for ischemic stroke patients

Figure 1. HBO-PC attenuated HT after MCAO in hyperglycemic rats dependent on activation of PPAR γ . (A) Representative pictures for TTC staining. (B) HBO-PC effectively reduced the infarct volume and PPAR γ inhibitor GW9662 abolished the effects at 24 hours after MCAO in hyperglycemic rats. (C) HBO-PC attenuated the hemorrhage volume significantly and GW9662 reversed the results of HBO-PC. * $p < 0.05$ vs. DX+Sham; # $p < 0.05$ vs. DX+MCAO; & $p < 0.05$ vs. DX+MCAO+HBO. N=6 for each group.

Figure 2.

HBO-PC decreased brain water content (A), improved neurological deficits (B) and showed tendency to reduce mortality (C) at 24 hours after MCAO. HBO-PC had no effects on blood glucose level (D). * $p < 0.05$ vs. DX+Sham; # $p < 0.05$ vs. DX+MCAO. $N = 6$ in each group in (A) and (B); $n = 12$ in each group in (D).

Figure 3. HBO-PC increased 15d-PGJ2 and PPAR γ at 24 hours after MCAO. (A) HBO-PC increased the level of 15d-PGJ2 in naïve rats 24 hours after the last HBO exposure. (B) The level of 15d-PGJ2 in sham and MCAO animals were relatively low, and HBO-PC significantly increased the level of 15d-PGJ2 at 24 hours after MCAO. (C) PPAR γ mildly increased 24 hours after MCAO and HBO-PC greatly upregulated the expression of PPAR γ . @ $P<0.05$ versus Naïve; * $p<0.05$ vs. DX+Sham; # $p<0.05$ vs. DX+MCAO. Naïve, Naïve+HBO-PC, and Sham, $n=6$; other groups, $n=12$.

Figure 4. HBO-PC suppressed inflammation by increasing PPAR γ at 24 hours after MCAO. (A) Representative Western blots of TNF- α and IL-1 β . (B) and (C) HBO-PC effectively suppressed the increase of TNF- α and IL-1 β , and administration of GW9662 before each HBO-PC removed the effects of HBO-PC. * $p < 0.05$ vs. DX+Sham; # $p < 0.05$ vs. DX+MCAO; & $p < 0.05$ vs. DX+MCAO+HBO. Sham, $n = 6$; other groups, $n = 12$.

Figure 5. HBO-PC increased ZO-1 and Occludin through activating PPAR γ . (A) Representative Western blots of ZO-1 and Occludin. (B) and (C) HBO-PC significantly increased ZO-1 and Occludin, and administration of GW9662 ablished the effects of HBO-PC. * $P < 0.05$ versus DX+Sham, # $P < 0.05$ versus DX+MCAO, & $P < 0.05$ versus DX+MCAO+HBO. Sham, $n = 6$; other groups, $n = 12$.

Figure 6. HBO-PC inhibited the activity of MMP-9 by activation of PPAR γ at 24 hours after MCAO. (A) Representative bands for zymography. (B) and (C) Statistical analysis for MMP-9 and MMP-2. HBO-PC remarkably decreased the activity of MMP-9, and had no effects on the activity of MMP-2; PPAR γ inhibitor GW9662 increased the activity of MMP-9 after HBO-PC. * $p < 0.05$ vs. DX+Sham; # $p < 0.05$ vs. DX+MCAO; & $p < 0.05$ vs. DX+MCAO+HBO. Sham, $n = 6$; other groups, $n = 12$.