Original article

TRPV4 plays an important role in rat prefrontal cortex changes induced by acute hypoxic exercise

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ABSTRACT

Objective: This study aims to investigate the effects of TRPV4 on acute hypoxic exercise-induced central fatigue, in order to explore the mechanism in central for exercise capacity decline of athletes in the early stage of altitude training.

Methods: 120 male Wistar rats were randomly divided into 12 groups: 4 normoxia groups (quiet group, 5-level group, 8-level group, exhausted group), 4 groups at simulated 2500 m altitude (grouping as before), 4 groups at simulated 4500 m altitude (grouping as before), 10 in each group. With incremental load movement, materials were drawn corresponding to the load. Intracellular calcium ion concentration was measured by HE staining, enzyme-linked immunosorbent assay, immunohistochemistry, RT-qPCR, Fluo-4/AM and Fura-2/AM fluorescence staining.

Results: (1) Hypoxic 2–5 groups showed obvious venous congestion, with symptoms similar to normoxia-8 group; Hypoxic 2–8 groups showed meningeal loosening edema, infra-meningeal venous congestion, with symptoms similar to normoxia-exhausted group and hypoxic 1-exhaused group. (2) For 5,6-EET, regardless of normoxic or hypoxic environment, significant or very significant differences existed between each exercise load group (normoxic − 5 level 20.58 ± 0.66 pg/mL, normoxic − 8 level 23.15 ± 0.46 pg/mL, normoxic - exhausted 26.66 ± 0.71 pg/mL; hypoxic1-5 level 21.72 ± 0.43 pg/mL, hypoxic1-8 level 24.73 ± 0.69 pg/mL, hypoxic 1-exhausted 28.68 ± 0.48 pg/mL; hypoxic2-5 level 22.75 ± 0.20 pg/mL, hypoxic2-8 level 25.62 ± 0.39 pg/mL, hypoxic 2-exhausted 31.03 ± 0.41 pg/mL) and quiet group in the same environment(normoxic-quiet 18.12 ± 0.65 pg/mL, hypoxic 1-quiet 19.94 ± 0.43 pg/mL, hypoxic 2-quiet 21.72 ± 0.50 pg/mL). The 5,6-EET level was significantly or extremely significantly increased in hypoxic 1 environment and hypoxic 2 environment compared with normoxic environment under the same load. (3) With the increase of exercise load, expression of TRPV4 in the rat prefrontal cortex was significantly increased; hypoxic exercise groups showed significantly higher TRPV4 expression than the normoxic group. (4) Calcium ion concentration results showed that in the three environments, 8 level group (normoxic-8 190.93 ± 6.11 nmol/L, hypoxic1-8 208.92 ± 6.20 nmol/L, hypoxic2-8 219.13 ± 4.57 nmol/L) showed very significant higher concentration compared to quiet state in the same environment (normoxic-quiet 107.11 ± 0.49 nmol/L, hypoxic 1-quiet 128.48 ± 1.51 nmol/L, hypoxic 2-quiet 171.71 ± 0.84 nmol/L), and the exhausted group in the same environment (normoxic-exhausted 172.51 ± 3.30 nmol/L, hypoxic 1-exhausted 164.54 ± 6.01 nmol/L, hypoxic 2-exhausted 154.52 ± 1.80 nmol/L) had significant lower concentration than 8-level group; hypoxic2-8 had significant higher concentration than normoxic-8.

Conclusion: Acute hypoxic exercise increases the expression of TRPV4 channel in the prefrontal cortex of the brain. For a lower ambient oxygen concentration, expression of TRPV4 channel is higher, suggesting that TRPV4 channel may be one important mechanism involved in calcium overload in acute hypoxic exercise.

1. Introduction

In the early stage of altitude training, hypoxia-induced decline in exercise capacity prevented normal progression of training. Since the 1990s, researches on this issue have focused more on hypoxia-induced cardiopulmonary function and decreased oxygen utilization in muscles, but studies have already shown that acute hypoxia can lead to decreased blood oxygen saturation in brain tissue, resulting in a series of changes in central function which may concern declined exercise capacity (Imray et al., 2005; Andrew et al., 2009). In near-exhaustion exercise, the declined oxidation of the prefrontal lobes limits exercise performance until the exercise stops owing to reduced executive function (Imray et al., 2005). In exhaustive exercise in a hypoxic environment, prefrontal cortex has greater deoxygenation degree than the motor area and the premotor area (Andrew et al., 2009). The prefrontal cortex of the brain is the upper part of the motor area. Its main functions involve the formulation of exercise strategies and exercise plans, as well as selection of exercise programs. With important executive functions, the prefrontal cortex has extensive neural connections, complex structures and rich and complex two-way connections, etc., thus one of the most important areas of the brain (Suzuki et al., 2004; Bao, 2012; Ding, 2012). In addition, changes in blood flow and blood oxygen in the local area of the brain (hemodynamics in general) are closely related to the activation of nerve cells (Bao, 2012; Ding, 2012).

In the mechanism study on central fatigue, the theory of calcium overload has been a focus of attention. Intracellular calcium overload causes weakened nerve excitability, and results in blood flow redistribution by neurovascular coupling mechanism. Transient Receptor Potential Vanilloid 4 (TRPV4) channel plays an important role in regulating intracellular calcium ions. It is mainly distributed in hippocampus, cerebral cortex, thalamus and cerebellum (Huang and Hu, 2017). Mechanical stimulation like cell swelling and chemical stimulation of arachidonic acid metabolites can activate TRPV4 channel (Huang and Hu, 2017; Huang et al., 2017). Therefore, the study aims to explore the effects of TRPV4 on acute hypoxic exercise-induced central fatigue, in order to explore the mechanism in central for exercise capacity decline of athletes in the early stage of altitude training, and provide a new nutritional improvement target for it.

2. Research objects and methods

2.1. Research objects

In the study, 120 male Wistar rats weighing 280–300 g purchased from Beijing Weitong Lihua Experimental Animal Center were taken as research objects. The study was approved by the Ethics Committee (Approval No. 2016010). The rats were kept in cages, with room temperature maintained at 20–24 °C, relative humidity controlled at 40–60%, and illumination of 12 h light/12 h light out per day. The national standard rodent animals were fed with conventional fodder. Animal drinking water and diet were not controlled (Xu et al., 2017). The rats were randomly divided into 12 groups (Fig. 1): 10 in the normoxic quiet group, 10 in the normoxic 5-level group, 10 in the normoxic 8-level group, 10 in
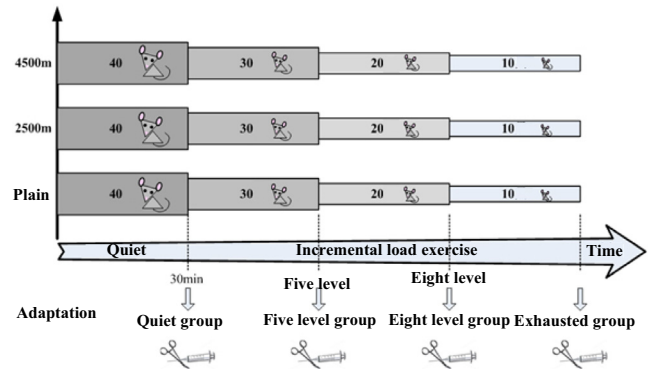

Fig. 1. Experimental flow.

the normoxic exhausted group, 10 in the 2500 m quiet group, 10 in the 2500 m 5-level group, 10 in the 2500 m 8-level group, 10 in the 2500 m exhausted group, 10 in the 4500 m quiet group, 10 in the 4500 m 5-level group, 10 in the 4500 m 8-level group, and 10 in the 4500 m exhausted group.

2.2. Main experimental reagents

The anti-TRPV4 antibody ab39260 (Abcam), RNAprep Pure Tissue Kit (TIANGEN BIOTECH), TRPV4 and IP3R primers (Sangon Biotech (Shanghai) Co., Ltd.), Prime ScriptTM RT MasterMix kit (Takara), Rat 5,6-EET ELISA kit (MM-0703R1), fluorescent calcium indicator Fluo-4/AM F14201 (Invitrogen by Thermo Fisher Scientific), fluorescent calcium indicator Fura-2/AM (DojindoMolecular Technologies, Inc. (Shanghai)).

2.3. Main experimental instruments

(See Table 1).

2.4. Rats adaptive treadmill training program

The rats were adaptively trained on the treadmill for 5 days at a speed of 10 m/min, 15 min per day. At 1-day interval after the end of the adaptive treadmill training period, microelectrode insertion was performed, and subsequent experiments were carried out.

Table 1
Main experimental instruments.

Instrument Name	Model
Microscope	Olymbus BX43
Ultra-micro UV spectrophotometer	Nano drop 2000c thermo scientific
Real-time fluorescence PCR instrument	ABI7500
Reverse transcription PCR instrument	Bio rad iCycle
Microplate reader	Labsystems Multiskan MS 352
Microplate Washer	Thermo Labsystems
Waterproof thermostatic water bath	GNP-9080
Vibratome	NVSLM1
Laser Scanning Confocal Microscope	Leica SP8

Table 2
Rat incremental load exercise program.

Stage	Slope (°)	Speed (m/min)	Duration (min)
1	10	5	4
2	10	5	3
3	10	5	3
4	10	5	3
5	10	5	3
6	10	5	3
7	10	5	3
8	10	5	3
9	10	5	3
10	10	5	3

2.5. Rat incremental load exercise program

The incremental load exercise protocol in rats was based on Leandro's test model on the maximal oxygen uptake of Wistar rats. In this study, incremental load exercise experiment was performed based on Leandro's test model on maximal oxygen uptake of Wistar rats. Incremental load exercise was taken according to Table 2, with slope maintained at 10°. With initial speed at 5 m/min, the speed was maintained for 4 min, and then added by 5 m/min every 3 min till exhaustion. The maximum speed was 50 m/s (Xu et al., 2017; Leandro et al., 2007). When the rat is unable to continue running on the treadmill under the condition of electrical stimulation, it is judged to be exhausted.

2.6. Material drawing

Anesthesia was administered intraperitoneally with 3% sodium pentobarbital (0.25 ml/100 g body weight). After anesthesia, blood was drawn from abdominal aorta with brain tissue quickly removed.

2.7. Test indicators and methods

2.7.1. Paraffin section and HE staining
Part of the prefrontal cortex was cut and placed in 4% paraformaldehyde for paraffin sectioning. The fixed forehead portion was dehydrated with gradient alcohol, made transparent with xylene, dipped in wax, embedded, and paraffin-sliced. Each brain was sliced continuously, and one slice was taken per 5 slices, with a total of 6 slices taken for conventional hematoxylin and eosin staining, observation under olympus microscope BX43 and photography (Huang et al., 2017).

2.7.2. Immunohistochemistry

(1) Paraffin sections are dewaxed by xylene, dehydrated with gradient alcohol, and descended to distilled water.
(2) Washed by PBS 3 times, 5 min each time.
(3) 3% hydrogen peroxide action at room temperature away from light for 20 min.
(4) Washed with distilled water and washed 3 times by PBS, 5 min each time.
(5) The 5% bovine serum albumin blocking solution was applied at room temperature for 30 min.
(6) After dumping the blocking solution, the diluted primary antibody (1:200-fold dilution of rabbit-derived TRPV4 polyclonal antibody) was added dropwise for 4 °C overnight stay.
(7) Washed by PBS 3 times, 5 min each time.
(8) Add reagent 1 (polymer adjuvant) for action at 37 °C for 30 min.
(9) Repeat step 7.

(10) Reagent 2 (horseradish peroxidase multimer-labeled goat anti-rabbit IgG) was added dropwise and allowed to act at 37 °C for 30 min.
(11) Washed by PBS 3 times, 5 min each time.
(12) Add DAB coloring solution dropwise, keep at room temperature away from light for 2–10 min, and control the color developing time under the microscope.
(13) Washed by distilled water, applied with hematoxylin dye solution for 5 min.
(14) Washed by distilled water, color separated with 1% hydrochloric-alcohol solution for 10 s, and blued with 0.1 M pH 7.4 PBS for 15 min.
(15) Dehydrated by gradient alcohol, after made transparent with xylene, sealed with neutral balsam and observed under the microscope. Meanwhile, a staining control was set. That is, the primary antibody was replaced by PBS. Rating method: 0 = no positive cells; 1 = 1–5 positive cells; 2 = 6–10 positive cells; 3 = 11–15 positive cells; 4 = 16–20 positive cells; 5 = more than 20 positive cells.

2.7.3. Test of relative content of TRPV4 and IP3R mRNA
2.7.3.1. Primer design.
In primer design, primer sequence of corresponding genes was designed by querying NCBI's Prime-Blast search and Oligo7.0 software detection. The synthesis of all primers was carried out in Sangon Biotech (Shanghai) Co., Ltd. The upstream and downstream primers were duplicated and stored at −20 °C until use (Xu et al., 2017).

TRPV4(5′ − 3′)FORWARD:CAGCAAGATCGAGAACCGCCAT
REVERSE: CGAACTTACGCCACTTGTCCCT

2.7.3.2. Total RNA extraction and concentration determination.
Part of the prefrontal cortex was placed in RNA Later and placed at −20 °C for RNA extraction.

RNA extraction was performed according to RNAprep Pure Tissue Kit (TIANGEN BIOTECH) instructions to extract total RNA from the rat prefrontal cortex. The specific steps (Xu, 2014) are as follows:

(1) Homogenization: 300 μl of lysate was added to every 10–20 mg of tissue, and homogenized thoroughly with a homogenizer; then 590 μl of RNase-Free dd H_2O and 10 μl of Proteinase K were added to the homogenate, mixed and placed at 56 °C for 10–20 min processing.
(2) Centrifuge at 12,000 rmp (∼13400g) for 2–5 min, and then take the supernatant.
(3) Absolute ethanol at 0.5 times of supernatant volume was slowly added to the extracted supernatant and shaken up. The solution and precipitate were transferred into the adsorption column CR3 (adsorption column was placed in the collection tube) and centrifuged at 12000 rmp (∼13400g) for 30–60 s. After waste liquid in the collection tube was discarded, the adsorption column was returned to the collection tube.
(4) The 350 μl of deproteinized solution RW1 was added to the adsorption column CR3, centrifuged at 12000 rpm (∼13400g) for 30–60 s. The waste liquid was discarded, and the adsorption column was returned to the collection tube.
(5) Preparation of DNase 1 working solution: Take 10 μl of DNase 1 stock solution into a new RNase-Free centrifuge tube, add 70 μl of RDD solution, and mix gently.
(6) The 80 μl of DNase 1 working solution was added to the center of the adsorption column CR3 and kept at room temperature for 15 min.

(7) Add 350 μl of deproteinized solution RW1 to the adsorption column CR3, centrifuge at 12,000 rmp (~13400g) for 30–60 sec, discard the waste liquid, and put the adsorption column back into the collection tube.

(8) The 500 μl of wash liquid RW was added to the adsorption column CR3, placed at room temperature for 2 min, centrifuged at 12000 rpm (~13400g) for 30–60 sec. The waste liquid was discarded, and the adsorption column CR3 was placed back in the collection tube.

(9) Repeat step 8.

(10) Centrifuge for 2 min at 12,000 rmp (~13400g) and discard the waste liquid. The adsorption column CR3 was placed at room temperature for a few minutes to thoroughly dry the residual wash liquid in the adsorbent material.

(11) The adsorption column CR3 was transferred to a new RNase-Free centrifuge tube, and 30–100 μl of RNase-Free dd H_2O was added to the middle of the adsorption membrane in suspension, kept at room temperature for 2 min, and centrifuged at 12000 rpm (~13400g) for 2 min to obtain RNA solution (Xu, 2014).

The total RNA concentration was measured by an ultraviolet spectrophotometer at absorbance values of 260 nm and 280 nm and the concentration was calculated. The purity of total RNA was detected on an agarose gel (BIO-RAD electrophoresis apparatus). All ratios of A260/280 were between 1.8 and 2.0, indicating purity of the extracted RNA.

2.7.3.3. RT-PCR reaction. The reverse transcription process was carried out according to the instructions of Prime Script™ RT Master-Mix Kit (Takara) to obtain cDNA. The specific operations are as follows:

(1) Prepare RT reaction solution according to the following components (see Table 3).

(2) Perform reverse transcription reaction after gentle mixture, with conditions shown as follows:
37 °C 15 min (reverse transcription reaction)
38 °C 5 s (reverse transcriptase inactivation reaction)
4 °C

The obtained RT reaction solution was added to the next Real Time PCR reaction system in an amount not exceeding 1/10 of the Real Time PCR reaction system.

(3) Prepare PCR reaction solution according to the following components (see Table 4)

(4) Perform Real Time PCR reaction

Table 3
Preparation of RT reaction solution.

Reagent	Usage amount
5 × PrimeScript RT Master Mix (Perfect Real Time)	2 μl
Total RNA(<500 ng/10μL reaction system)	400 ng
RNase Free dH₂O	10 μl

Table 4
Preparation of PCR reaction solution.

Reagent	Usage amount
SYBR Premix Ex Taq II (Tli RNaseH Plus) (2X)	10 μl
PCR Forward Primer (10 μM)	0.8 μl
PCR Reverse Primer (10 μM)	0.8 μl
RT reaction solution	2 μl
dH₂O	6 μl
Tatal	20 μl

Amplify the standard procedure using a two-step method:
The first step: pre-change

Reps: 1
95 °C 30 s

The second step: PCR reaction

Reps: 40
95 °C 5 s
60 °C 30–34 s

After completion of the reaction, the amplification curve and dissociation curve were confirmed, and the relative quantification was carried out by $2\text{-}^{\triangle\triangle CT}$ method.

2.7.4. 5,6-EET enzyme-linked immunosorbent assay
2.7.4.1. Sample processing. The 1 g of prefrontal cortex tissue was weighed, and 9 g of pH 7.2–7.4PBS was added and thoroughly homogenized with a homogenizer. Centrifuge for 20 m (2000–3000 rpm), carefully collect the supernatant and dispense for testing.

2.7.4.2. Determination of 5,6-EET. In this experiment, MM-0703R1 rat 5,6-EET ELISA kit was used. The specific operation steps (Zhang, 2016) are as follows:

(1) Dilution of standard substance: The homogeneous standard substance was diluted according to the instructions, and dispensed to small tubes.

(2) Sample adding: Set blank hole (the operation steps were consistent except that the blank hole was not added with sample and conjugate reagent), standard hole and sample hole. The standard hole on enzyme-labeled plate was accurately added with 50 μl sample. The sample hole was first added with 40 μl sample dilution and then added with 10 μl sample to be tested (the final dilution of the sample was 5 times). During sample adding, note that the sample should be added to the bottom of the ELISA plate. Avoid touch with the wall and gently shake it to mix.

(3) Incubation: After sealing the plate hole with a microplate sealer, incubate at 37 °C for 30 min.

(4) Fluid preparation: The 30 times concentrated washing solution was diluted 30 times with distilled water for use.

(5) Washing: Remove the microplate sealer as carefully as possible, discard the liquid, and dry it, then fill each hole with the washing solution, leave it for 30 s, discard it, repeat 5 times, then pat it dry.

(6) Enzyme supplementation: Add 50 μl of conjugate reagent to each hole, except for blank hole.

(7) Incubation: The operation method is the same as 3.

(8) Washing: The operation method is the same as 5.

(9) Color development: Add color developer A: 50 μl to each hole, then add color developer B: 50 μl, gently shake and mix, and let it stand for 10 min at 37 °C in the dark.

(10) Termination: The 50 μl of stop solution was added to each hole, and the reaction was terminated (the sign of termination was blue turned to yellow).

(11) Measurement: Zero setting was performed using a blank hole, and the absorbance (OD value) in each hole was measured in turn at a wavelength of 450 nm. The measurement procedure should be performed within 15 min after the addition of stop solution (Zhang, 2016).

2.7.5. Preparation of living brain slices and Fluo-4/AM fluorescent staining

2.7.5.1. Preparation of artificial cerebrospinal fluid. Artificial cerebrospinal fluid (ACSF) 1000 ml was prepared with the main components: 124 mmol/L NaCl, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, 1.23 mmol/L NaH$_2$PO$_4$, 3 mmol/L KCl, 26 mmol/L NaHCO$_3$ and 10 mmol/L glucose.

2.7.5.2. Preparation of living brain slices. The artificial cerebrospinal fluid was ventilated with a mixed gas of 95% O$_2$ + 5% CO$_2$ for half an hour before the preparation of living brain slices to achieve saturation. The repaired brain tissue was placed on a microtome, and the prefrontal cortex was cut into 300 μm thick brain tissue sections, and placed in an oxygen-saturated artificial cerebrospinal fluid for reoxygenation for 30 min.

2.7.5.3. Fluo-4/AM fluorescent staining. The brain tissue section after reoxygenation was placed in prepared Fluo-4/AM coloring agent (Invitrogen fluorescent calcium indicator Fluo-4/AM F14201) solution in the dark for 40 min' 37 °C water bath, during which gas mixture of 95% O$_2$ + 5% CO$_2$ was continuously ventilated in the dark. The brain slices were then soaked in cerebrospinal fluid ventilated with the gas mixture as before for a while, taken out and placed in a laser confocal culture dish.

Then, the intracellular calcium staining of each group of brain slices was observed by laser confocal microscopy.

2.7.6. Fura-2/AM fluorescent staining for determination of intracellular calcium ion concentration

Fura-2/AM was purchased from Dojindo Molecular Technologies, Inc. The reagent preparation and the experimental procedures were performed with reference to the product manual and experimental methods of Li et al. (1991), Grynkiewicz et al. (1985), An et al. (2002) and Xu et al.

2.7.6.1. Preparation of reagents.
(1) Hank's solution (Glucose 6, KCl 5, NaCl 137, Na$_2$HPO$_4$ 0.3, MgSO$_4$ 1, CaCl$_2$ 1, 4-Hydroxyethylpiperazine Ethane Sulfonic Acid (HEPES) 10, unit m mol/L; pH 7.4) and D-Hank's solution (Glucose 6, KCl 5, NaCl 137, Na$_2$HPO$_4$ 0.3, HEPES10 unit mmol/L; pH 7.4) was self-prepared with three distilled water.
(2) Preparation of mother liquor: 50 μg of Fura 2-AM powder was dissolved in 49.9 μl of dimethyl sulfoxide (DMSO) to prepare a 1 mmol/L Fura 2-AM mother liquor for sealed storage at −20 °C in the dark.
(3) Preparation of Fura-2/AM working solution: 1–5 mmol/l of Fura-2/AM mother liquor was diluted with Hank's solution to prepare 1–5 μmol/l Fura-2/AM working solution.

2.7.6.2. Experimental steps. The prefrontal cortex tissue of the brain was washed 3 times in icy D-Hank's solution, and the brain membrane and blood vessels were removed. The isolated cerebral cortex tissue was chopped, and appropriate pancreatin with 0.1% mass fraction was added and evenly mixed. Stir in a 37 °C water bath and digest for 10 min; then the tissue block was quickly transferred to ice-cold stop solution (with Hank's solution containing 10% calf serum) to stop digestion. The tissue block was beaten into cell suspension, screened with 200 mesh sieve to collect cell suspension, with cell density controlled between 106/ml-107/ml. Then, the cell suspension was placed in a 37 °C water bath for 5 min, then Fura -2/AM (with the final concentration of 5 μmol/L) was added, and after 45 min constant temperature water bath at 37 °C, it was taken out and centrifuged at 800 r/min for 5 min

before discarding the supernatant. Then rinse it twice with Hank's solution containing 0.2% bovine serum albumin to remove extracellular redundant Fura-2/AM coloring agent (Li et al., 1991; An et al., 2002; Xu et al.).

Cell viability was determined by trypan blue cell rejection. When the cell viability was greater than 95%, the measurement was performed on the machine. The cell suspension was warmed for 5 min before each measurement (Li et al., 1991; An et al., 2002; Xu et al.). Cell density analysis in cell counting was performed using Image Pro 6.0 software.

Measurement was performed in a fluorescence spectrophotometer with an emission wavelength of 510 nm, an excitation wavelength of 340 nm and 380 nm, and a grating of 5 nm. Finally, the maximum fluorescence value Rmax (obtained by adding 10% Triton-X-100) and the minimum fluorescence intensity value Rmin (obtained by adding EGTA at a concentration of 400 mmol/L) were determined.

Intracellular free Ca^{2+} concentration

$$= K_d(F_0/F_s)(R − R_{min})/(R_{max} − R)$$

K_d value in the formula is 224 nmol/l, F_0 and F_s represent the fluorescence intensity measured when Ca^{2+} is zero and saturated, respectively, R is the experimentally observed fluorescence ratio, and R_{max} and R_{min} represent the maximum and minimum fluorescence ratios, respectively (Li et al., 1991; An et al., 2002; Xu et al.).

2.8. Statistical methods

All data analysis was done in SPSS for Windows 19.0 software package. Two-way analysis of variance was performed on each indicator data (one factor was test environment and one factor was exercise intensity). Based on the interaction effect test, the two factors had no interaction effect, so the follow-up comparison (Post hoc) was performed by Tukey test (Imray et al., 2005; Zhang, 2016; Liu, 2015). The significance level was determined to be $P < 0.05$, and the very significant level was determined to be $P < 0.01$.

3. Research results

3.1. HE staining results of prefrontal cortex under incremental load exercise in different oxygen concentration environments

The brain tissue changes of each group are shown in Fig. 2:

Normoxic group: edema was observed in the 5-level mice brain tissue, and the gap between the blood vessels and the surrounding tissues was widened; edema and hemangiectasis, congestion were observed in the 8-level brain tissue; infra-meningeal venous congestion was observed in the exhausted group.

Hypoxic group 1 (2500 m group): small blood vessel dilatation and congestion were observed in the 5-level brain tissue, gliocyte neurotropic phenomenon and venous congestion were observed in 8-level brain tissue; meningeal loosening edema, infra-meningeal and cortical venous congestion were observed in the exhausted group.

Hypoxic group 2 (4500 m group): small blood vessel dilatation and congestion were observed in the quiet group; venous congestion and gliocyte neurotropic phenomenon were observed in 5-level brain tissue; gliocyte neurotropic phenomenon and infra-meningeal venous congestion were observed in 8-level brain tissue; loose intercellular tissues, disordered nerve cells, infra-meningeal congestion and hemorrhage and lymphocytic infiltration were observed in the exhausted group (Huang et al., 2017).
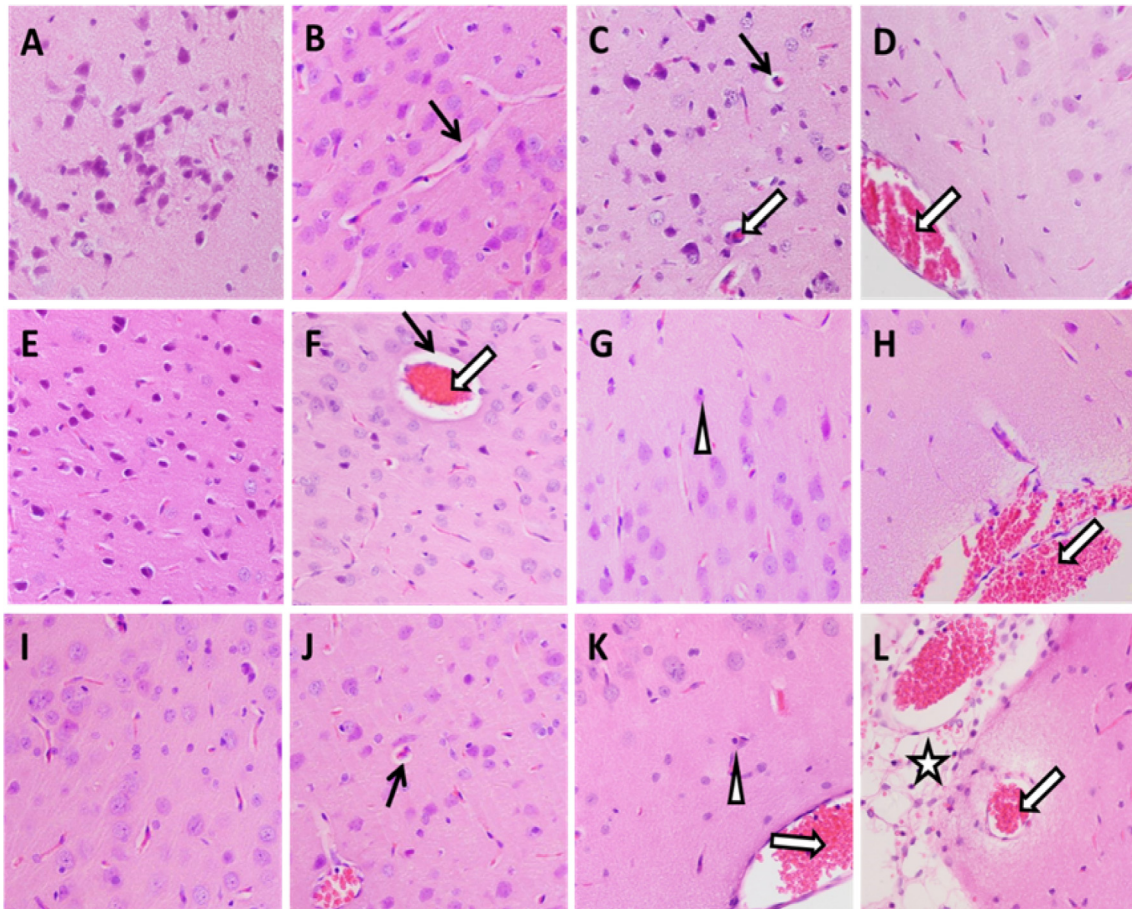
Fig. 2. HE staining of the prefrontal cortex of the brain (HE 40×). Note: Black arrow indicates edema, empty triangle indicates congestion, triangle indicates neurotropic phenomenon, and pentagram indicates loose structure and occasional lymphocytic infiltration. (A) Normoxic-quiet group, (B) normoxic-5-level group, (C) normoxic-8-level group, (D) normoxic-exhausted group, (E) hypoxic 1-quiet group, (F) hypoxic 1–5 level group, (G) hypoxic 1–8 level group, (H) hypoxic 1-exhausted group, (I) hypoxic 2-quiet group, (J) hypoxic 2–5-level group, (K) hypoxic 2–8-level group, (L) hypoxic 2-exhausted group.

3.2. Immunohistochemical results of TRPV4 channel protein in prefrontal cortex under incremental load exercise in different oxygen concentration environments

As shown in Figs. 3 and 4, TRPV4 positive signal is indicated in brownish yellow. Regardless of normoxic or hypoxic environment, the expression of TRPV4 in the rat prefrontal cortex increases significantly with the increase of exercise intensity. The 5, 8-level and exhausted groups in each environment show significantly higher expression than the quiet group in the same environment ($P < 0.01$).

In the quiet state, hypoxic 1-quiet group has significantly higherTRPV4expression than normoxic-quiet group ($P < 0.05$), and very significant difference ($P < 0.01$) is shown between hypoxic2- quiet group and normoxic-quiet group. In the 5-level state, very significant difference ($P < 0.01$) is shown between hypoxic 2–5 level group and normoxic-5 level group. In the 8-level state, significant difference ($P < 0.05$) is shown between hypoxic 1–8 level group and normoxic-8 level group, and very significant difference ($P < 0.01$) is shown between hypoxic 2–8 level group and normoxic-8 level group. In the exhausted state, significant difference ($P < 0.05$) is shown between hypoxic 1-exhausted group, hypoxic 2-exhausted group and normoxic-exhausted group. Significant difference is shown between hypoxic1-5 level group and hypoxic 1–5 level group, and significant difference is also shown between hypoxic 2–8 level group and hypoxic 1–8 level group (Huang et al., 2017).

3.3. Expression of TRPV4 mRNA in prefrontal cortex under incremental load exercise in different oxygen concentration environments

As shown in Figs. 5, 6, 7 and Table 5, in the normoxic environment, significant difference is shown in expression level of TRPV4 mRNA between 5-level group and quiet group, and very significant difference is shown between 8-level group and exhausted, quiet groups. Regardless of hypoxic 1 or hypoxic 2 environments, very significant difference is shown between in-motion, exhausted states and quiet state. No significant difference is shown in expression level of TRPV4 mRNA between hypoxic 1quiet state and normoxic quiet state, but very significant difference is shown between hypoxic 2 quiet state and normoxic quiet state. Significant or very significant differences are shown between in-motion and exhausted states of hypoxic environment and that in normoxic environment.

Note: Comparison within the group: * indicates comparison with quiet group in the same environment, $P < 0.05$, ** indicates comparison with quiet group in the same environment, $P < 0.01$. Comparison between groups: # indicates comparison with same-level normoxic group, $P < 0.05$; ## indicates comparison with same-level normoxic group, $P < 0.01$.

3.4. 5,6-EET test results of prefrontal cortex under incremental load exercise in different oxygen concentration environments

As shown in Fig. 8 and Table 6, significant or very significant difference is shown in 5,6-EET between each exercise load group and
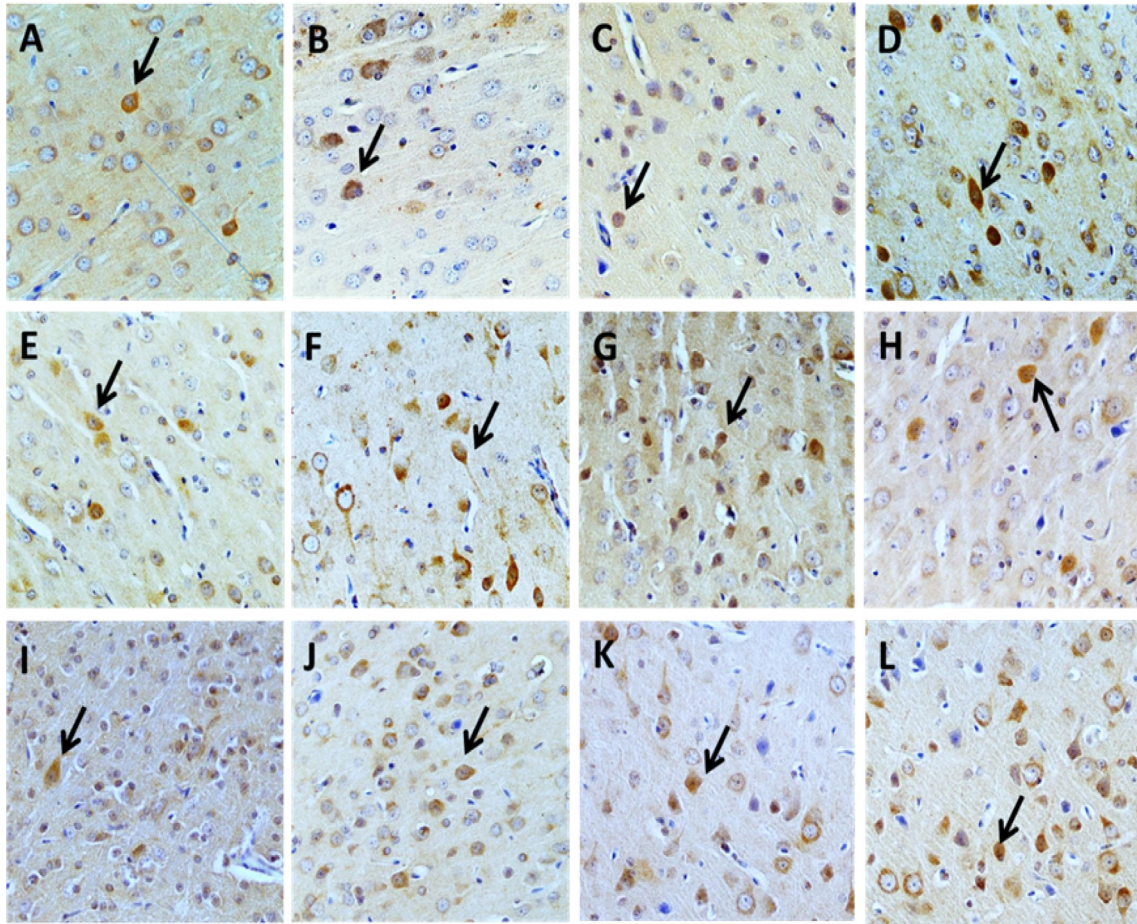
Fig. 3. Immunohistochemical staining of TRPV4 protein in the prefrontal cortex of the brain (IHC, 40×). Note: The arrow indicates TRPV4 positive signal. (A) Normoxic-quiet group, (B) normoxic-5-level group, (C) normoxic-8-level group, (D) normoxic-exhausted group, (E) hypoxic 1-quiet group, (F) hypoxic 1–5 level group, (G) hypoxic 1–8 level group, (H) hypoxic 1-exhausted group, (I) hypoxic 2-quiet group, (J) hypoxic 2–5-level group, (K) hypoxic 2–8-level group, (L) hypoxic 2-exhausted group.
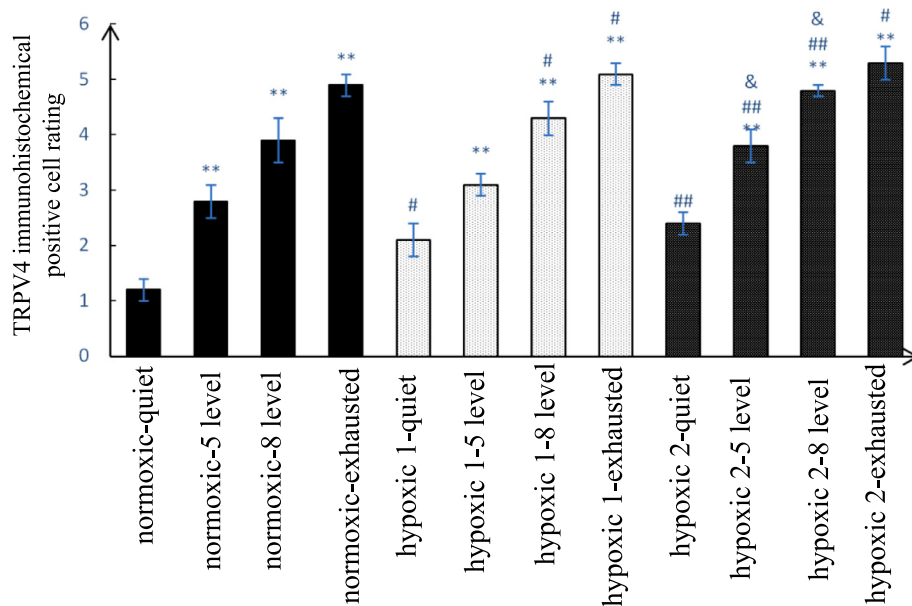
Fig. 4. Immunohistochemical results of TRPV4 channel in the prefrontal cortex of the brain. Note: Comparison within the group: * indicates comparison with quiet group in the hypoxic environment, $P < 0.05$, ** indicates comparison with quiet group in the hypoxic environment, $P < 0.01$. Comparison between groups: # indicates comparison with same-level normoxic group, $P < 0.05$; ## indicates comparison with same-level normoxic group, $P < 0.01$;& indicates comparison between hypoxic-2 level group and hypoxic 1-level group, $P < 0.05$.
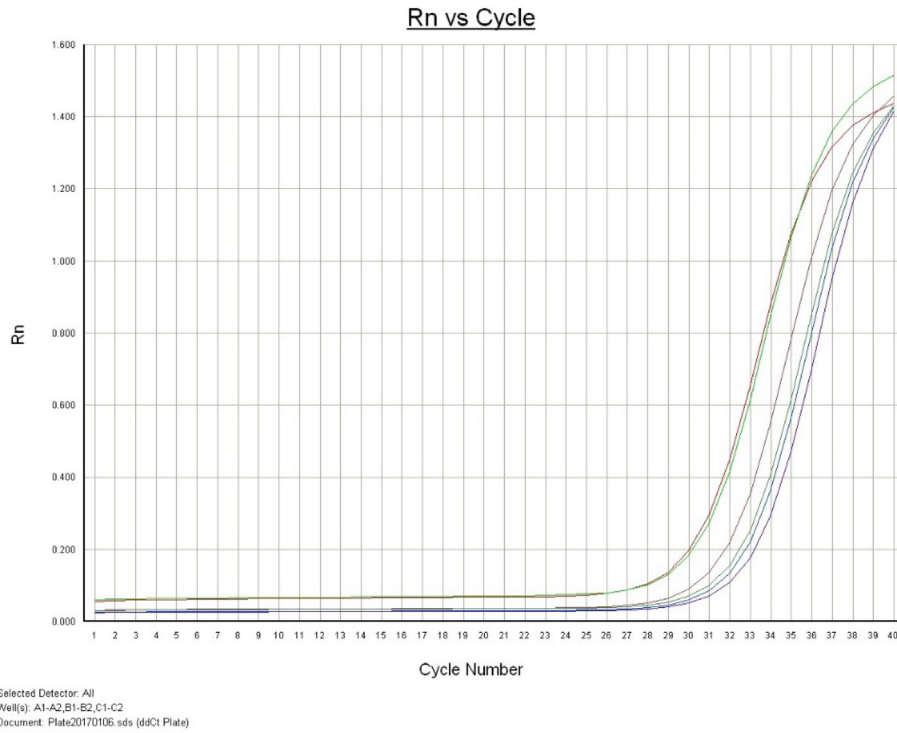
Rn vs Cycle

Selected Detector: All
Well(s): A1-A2,B1-B2,C1-C2
Document: Plate20170106.sds (ddCt Plate)

Fig. 5. PCR amplification curve of TRPV4 channel of rat prefrontal cortex.

Dissociation Curve

Detector = TRPV4, Tm = 60.0 °C
Well(s): A1-A2,B1-B2,C1,G1-G2
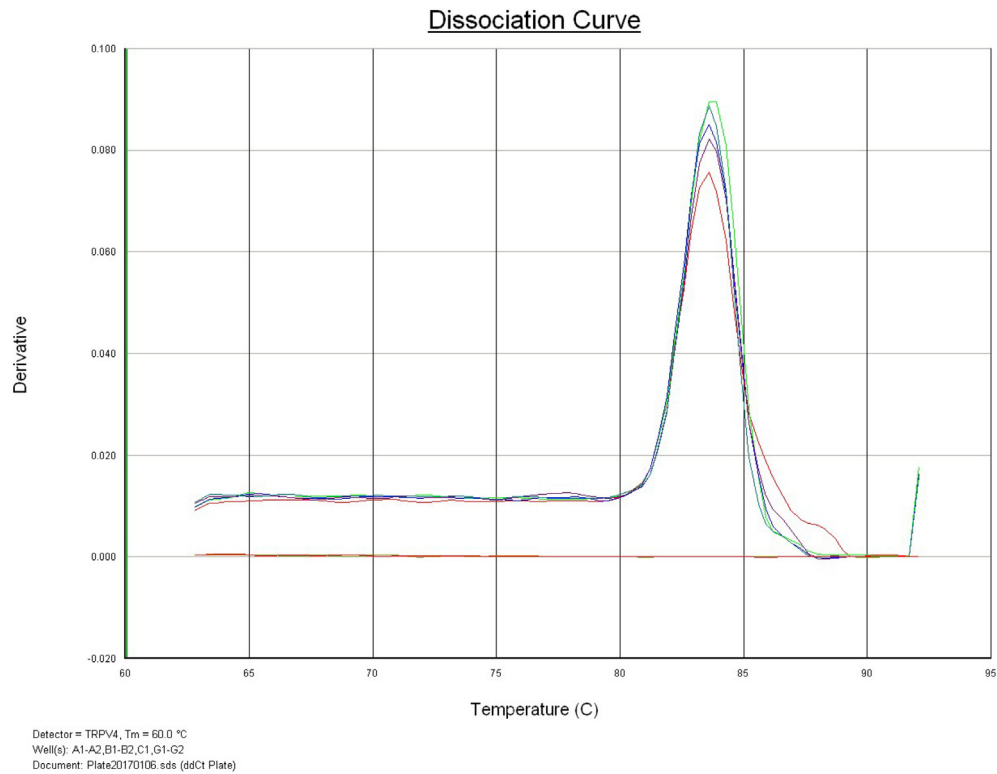Document: Plate20170106.sds (ddCt Plate)

Fig. 6. PCR dissociation curve of TRPV4 channel of rat prefrontal cortex.
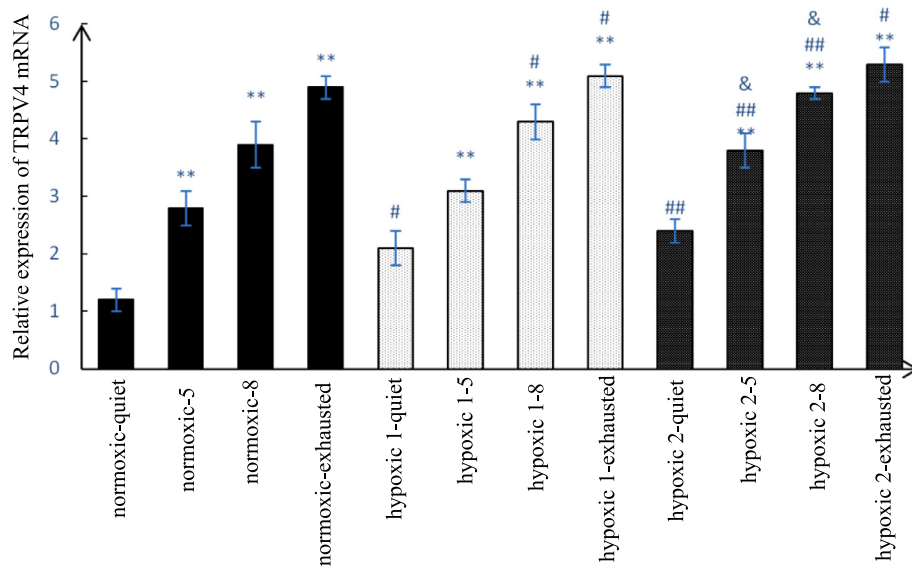
Fig. 7. Relative expression of TRPV4 mRNA in rat prefrontal cortex.

Table 5
Results of relative expression of TRPV4 mRNA.

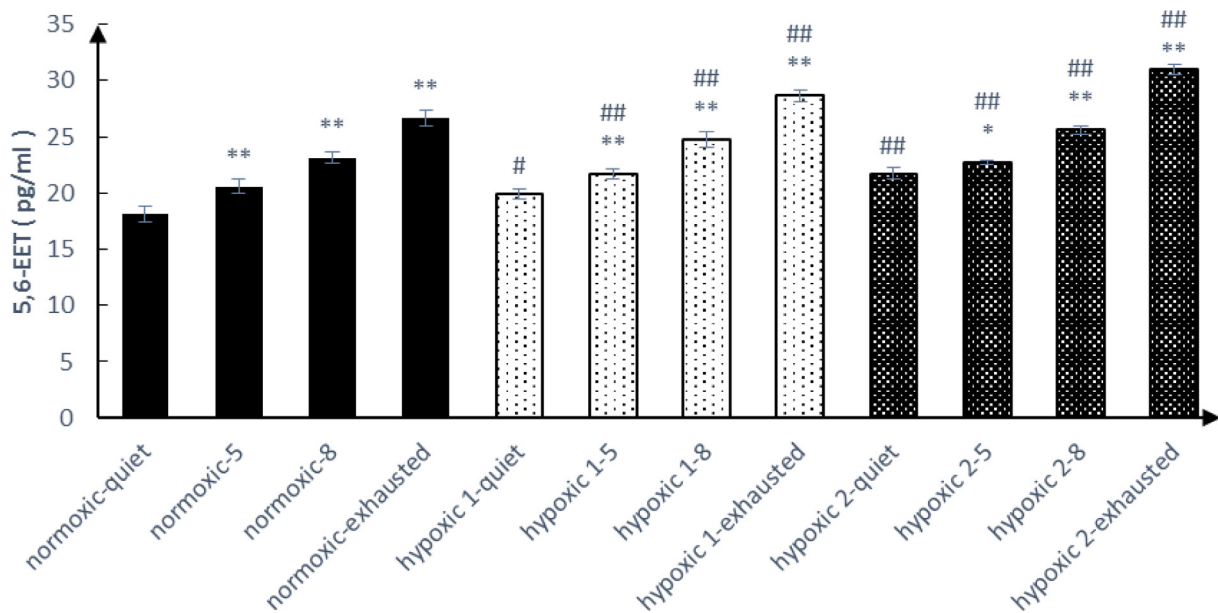| mRNA	Quiet	5-level	8-level	Exhausted
Normoxic group	0.53 ± 0.08	0.76 ± 0.11	0.91 ± 0.09	1.26 ± 0.16
Hypoxic 1 group	0.63 ± 0.12	1.20 ± 0.17	1.30 ± 0.14	1.49 ± 0.07
Hypoxic 2 group	0.73 ± 0.09	1.36 ± 0.07	1.50 ± 0.11	1.52 ± 0.12

Fig. 8. Changes in 5,6-EET concentration in the rat prefrontal cortex. Note: Comparison within the group: * indicates comparison with quiet group with low oxygen concentration, $P < 0.05$, ** indicates comparison with quiet group with low oxygen concentration, $P < 0.01$. Comparison between groups: # indicates comparison with same-level normoxic group, $P < 0.05$; ## indicates comparison with same-level normoxic group, $P < 0.01$.

Table 6
Changes in 5,6-EET concentration in the rat prefrontal cortex.

5,6-EET(pg/mL)	Quiet	5-level	8-level	Exhausted
Normoxic group	18.12 ± 0.65	20.58 ± 0.66	23.15 ± 0.46	26.66 ± 0.71
Hypoxic 1 group	19.94 ± 0.43	21.72 ± 0.43	24.73 ± 0.69	28.68 ± 0.48
Hypoxic 2 group	21.72 ± 0.50	22.75 ± 0.20	25.62 ± 0.39	31.03 ± 0.41
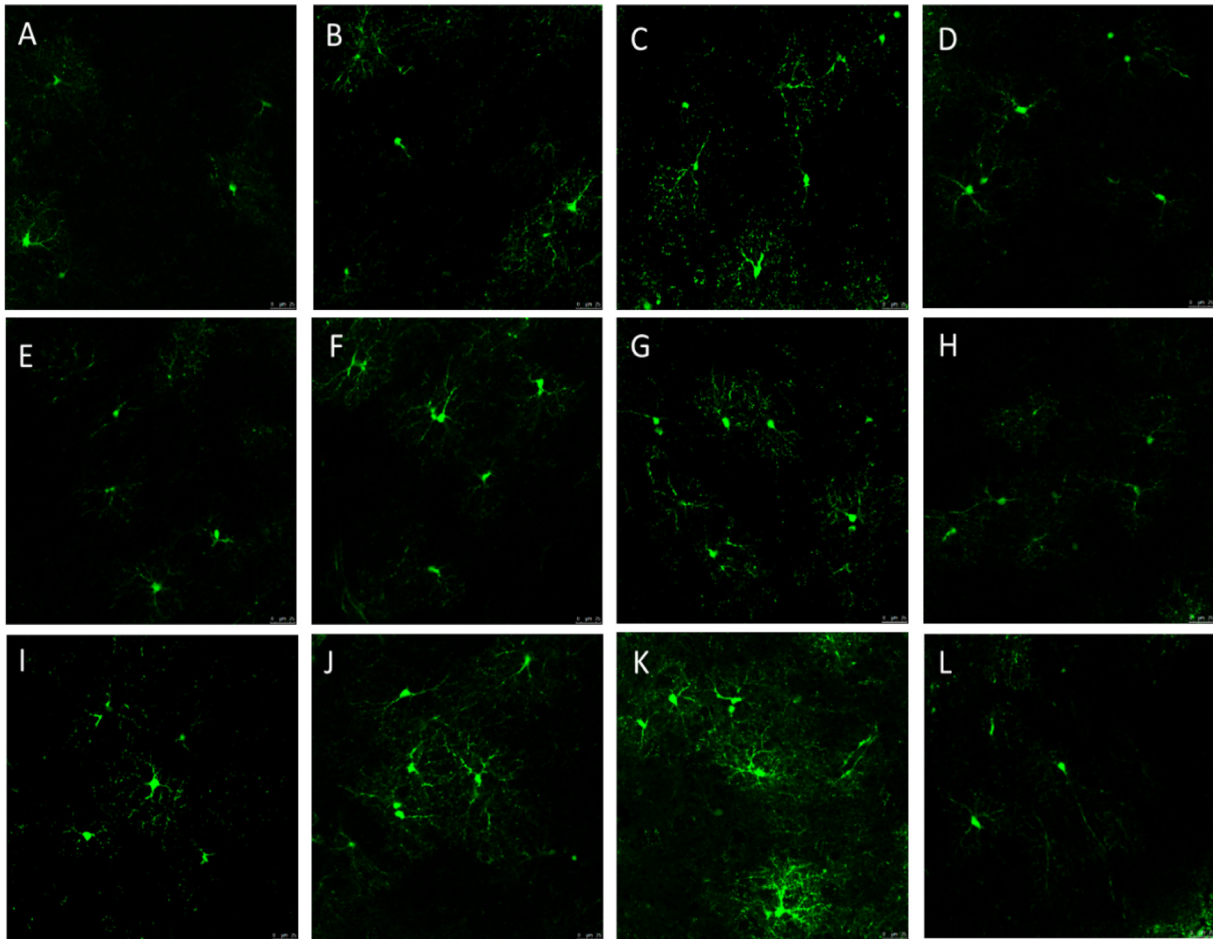
Fig. 9. Fluor-4/AM staining of calcium ion in rat prefrontal cortex. (A) Normoxic-quiet group, (B) normoxic-5-level group, (C) normoxic-8-level group, (D) normoxic-exhausted group, (E) hypoxic 1-quiet group, (F) hypoxic 1–5 level group, (G) hypoxic 1–8 level group, (H) hypoxic 1-exhausted group, (I) hypoxic 2-quiet group, (J) hypoxic 2–5-level group, (K) hypoxic 2–8-level group, (L) hypoxic 2-exhausted group.

quiet group in the same environment regardless of normoxic or hypoxic environment. The 5,6-EET level is significantly or very significantly higher in hypoxic 1 and 2 environments than same-load state in the normoxic environment.

3.5. Fluor-4/AM calcium ion staining of prefrontal cortex under incremental load exercise in different oxygen concentration environments

As shown in Fig. 9, the fluorescence intensity at 8-level is stronger than that under other load regardless of oxygen concentration environment. In same-level comparison between different oxygen concentration environments, fluorescence intensity is stronger for a lower oxygen concentration.

3.6. Quantitative detection of Fura-2/AM intracellular calcium in prefrontal cortex under incremental exercise load in different oxygen concentration environments

As shown in Figs. 10 and 11, in each oxygen concentration environment, calcium ion concentration under 8-level load is very significant higher than that in quiet state; and calcium ion concentration under 8-level load is significantly or very significantly higher than that in exhausted state in the same environment. Calcium ion concentrations in hypoxic 1- quiet group and hypoxic 2-quiet group are very significantly different from that in normoxic-quiet group. Calcium ion concentrations in hypoxic

1–5 level group and hypoxic 2–5 level group are very significantly different from that in normoxic-5 level group. Significant difference is shown between hypoxic 2–8 level group and normoxic- 8 level group; very significant difference is shown in calcium ion concentration between hypoxic 2-exhausted group and normoxic-exhausted group; significant difference is shown between hypoxic 1–8 level group and hypoxic 2–8 level group.

4. Discussion and analysis

4.1. TRPV4 channel immunohistochemistry and gene expression analysis

TRPV4 channel was first proposed by Liedtke et al. in 2000 (Liedtke et al., 2000). The human TRPV4 gene is present on 12q23-q24 chromosome and expressed on exon 15 (Anna et al., 2014). Five subtypes of TRPV4 have been identified, namely TRPV4-A-E. The phenomenon of protein retention, loss of oligomerization and channel inactivation in the cell endoplasmic reticulum can be caused by the deletion of subtypes B, C and E in N-terminal ankyrin repeat domain (ANK) (Arniges et al., 2006; Vazquez and Valverde, 2006). About 30% volume of the TRPV4 channel protein is located on the membrane, and about 70% volume is exposed intracellularly or extracellularly. Such structural feature provides great convenience for the interaction between the channel and the proteins inside and outside the cell so that channel is regulated (Verma et al., 2010).
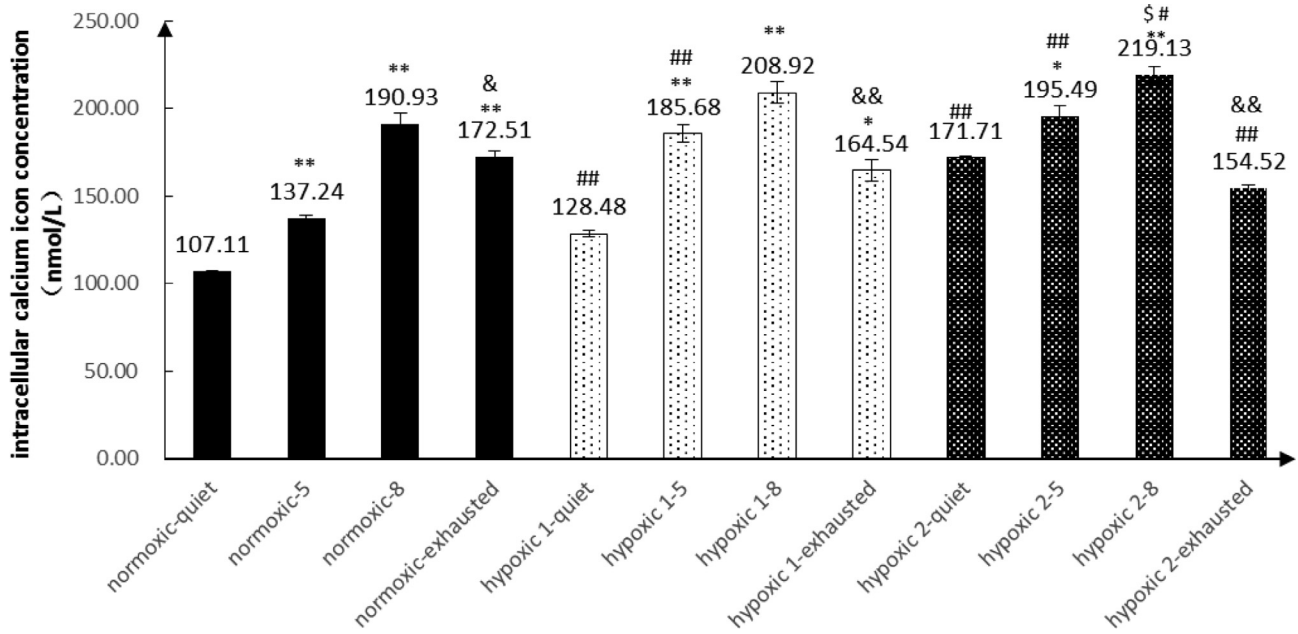
Fig. 10. Changes in intracellular calcium ion concentration. Note: Comparison within the group: * indicates comparison with quiet group in the same oxygen concentration environment, $P < 0.05$, ** indicates comparison with quiet group in the same oxygen concentration environment, $P < 0.01$, & indicates comparison between exhausted group and 8-level group in the same oxygen concentration, $P < 0.05$, && indicates comparison between exhausted group and 8-level group in the same oxygen concentration, $P < 0.01$. Comparison between groups: # indicates comparison with normoxic same-level group, $P < 0.05$; ## indicates comparison with normoxic same-level group, $P < 0.01$; $ indicates comparison between hypoxic 2–8 group and hypoxic 1–8 group, $P < 0.01$.
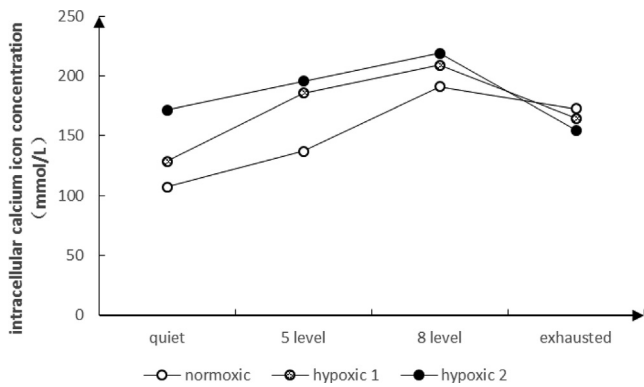
Fig. 11. Variation trend of intracellular calcium ion concentration.

Initially, TRPV4 channel was found to be an ion channel that can be activated by hypotension-induced swelling of cells (Liedtke et al., 2000; Nilius et al., 2001, 2004; Vennekens et al., 2008; Vriens et al., 2009). Subsequent studies have shown that TRPV4 can also be activated by warming, machinery, arachidonic acid or its metabolites (Plant and Strotmann, 2007; Kanju and Liedtke, 2016). TRPV4 channels are mainly distributed in cerebral cortex, hippocampus, thalamus, cerebellum, etc. (Shibasaki et al., 2007; Baets and De Jonghe, 2011; Liu et al., 2007; Lin et al., 2011; Chen et al., 2009, 2008a, 2008b).

The results of immunohistochemistry showed that the expression of TRPV4 in 5, 8 levels and exhaustive exercise intensity in normoxic environment was significantly higher than that in the quiet group, suggesting that exercise intensity of a certain load can lead to a significant increase in the expression of TRPV4 in the prefrontal cortex. With the increasing exercise intensity, the expression of TRPV4 is increased. Combined with the results of HE staining, the brain may be hypoxic due to exercise, then cell membrane tension is changed, activating TRPV4 channel. Meanwhile, the decrease of ambient oxygen concentration also

significantly increased the expression of TRPV4 in the rat prefrontal cortex. Even in a quiet state, the expression level of TRPV4 at 2500 m altitude was significantly higher than that of normoxia, and the expression level of TRPV4 at 4500 m altitude was very significantly increased. Significant difference also existed in the same-level comparison between the three groups, indicating that the hypoxic environment made a significant change on the expression of TRPV4 in exercise rats. The results of TRPV4 gene expression showed great similarity to the variation trend of immunohistochemistry results.

In case of cerebral ischemia and hypoxia, TRPV4 channel is activated mainly through the following two aspects: on the one hand, cerebral ischemia and hypoxia cause cell edema, and thus activate the TRPV4 channel by changing the mechanical tension of the cell membrane; on the other hand, cerebral ischemia and hypoxia cause energy metabolism disorder, producing a large amount of arachidonic acid (AA) which activates TRPV4 channel through its metabolite 5,6-EET (Huang and Hu, 2017; Li, 2014).

4.2. Analysis of HE staining

HE staining results demonstrated that the phenomenon of venous congestion was obvious at 5 level for 4500 m altitude, showing symptoms similar to that at 8 level in normoxic exercise; at 8 level for 4500 m altitude, meningeal loosening edema, infra-meningeal venous congestion were shown, with symptoms similar to that in normoxic exhausted state and exhausted state at 2500 m altitude. With the decrease of ambient oxygen concentration, hypoxic stimulation led to more obvious celluar swelling, venous hemorrhage and infra-meningeal venous hemorrhage in rat prefrontal cortex, and the phenomenon of cell swelling and hemangiectasis were advanced in the hypoxic environment (Huang et al., 2017). Studies have found that changes in mechanical gating factors such as cell edema during brain ischemia-reperfusion can lead to over-activation of TRPV4 receptors (Li, 2014). The main reason may be that cell edema changes cell membrane tension, TRPV4, a

pressure-sensitive channel, is then activated. Combining TRPV4 immunohistochemistry results, very significant difference is shown inTRPV4 expression between 5,8 levels, exhausted group in each oxygen concentration environment and quiet group, indicating that exercise-induced cell edema can significantly increase TRPV4 expression level. However, in hypoxia and quiet state without cell swelling, still significantly increased expression of TRPV4-may not be explained by the theory of mechanical gating. Therefore, it is speculated that increased secretion of certain chemicals caused by hypoxia may also affect TRPV4 channel activation.

4.3. Analysis of 5,6-EET results

The 5,6-EET is one metabolite of arachidonic acid (AA). The brain cell membrane is rich in unsaturated fatty acids, and free AA is decomposed by phospholipase C and phospholipase A2. Free AA is released from phospholipid pool on the cell membrane when the cell membrane is subjected to various stimuli, resulting in increased concentration and conversion into various metabolites, one of which is 5,6-EET (Liu, 1990). Studies have confirmed that 5;6 -EET activates TRPV4 channel (Plant and Strotmann, 2007).

The results of this study showed that significant or very significant differences existed in 5,6-EET between each exercise load group and quiet group in the same environment regardless of normoxic or hypoxic environment. Compared with the same-level load state of normoxic environment, 5,6-EET level was significantly or significantly increased in hypoxic 1 and 2 environment, indicating that hypoxic environment can significantly affect prefrontal cortex 5,6-EET level in exercise rats. Combining the results of TRPV4 immunohistochemistry, the expression level of TRPV4 channel was significantly higher in quiet state at 2500 m and 4500 m hypoxic environment than normoxic quiet group. The 5,6-EET in each oxygen concentration environment and each exercise level showed a change law very similar to the immunohistochemical results of TRPV4. Therefore, it is concluded that the increased expression of TRPV4 in prefrontal cortex of exercise rats in hypoxic environment may concern the increased secretion 5,6-EET by arachidonic acid under hypoxia stimulation which then activates the TRPV4 channel.

4.4. Analysis of intracellular calcium ion in prefrontal cortex during acute hypoxic exercise

In this study, two methods were used to detect intracellular calcium ion changes: one was Fluo-4/AM calcium ion fluorescent probe staining, and biopsy was stained. Fluo-4/AM is a commonly used calcium ion fluorescent probe. Fluo-4/AM is able to enter the cell through the cell membrane. Upon entry into the cell, the AM group in Fluo-4/AM is hydrolyzed to produce a separate Fluo-4. Fluo-4 has a strong affinity with free Ca^{2+}, and when combined with calcium ions, it can produce fluorescence under certain laser light, thus observed by laser confocal microscopy (Liu, 2015). This method is a semi-quantitative analysis method which can only be used for self-control of the same sample. In this study, Fluo-4/AM staining and viewing were adopted to intuitively visualize the changes of calcium ions in the prefrontal cortical nerve cells under different loads of normoxia and hypoxia. The other was Fura-2/AM calcium ion fluorescence staining, which can be used to measure intracellular free calcium ion concentrations in tissue blocks, organs, cultured monolayer adherent cells and platelets. Fura-2/AM, as a fluorescent indicator, binds to intracellular free calcium ions, and free form of Fura-2/AM requires a longer wavelength for excitation than the calcium-bound Fura-2/AM. By deriving the ratio of fluorescence intensity at two excitation wavelengths, the ratio of calcium-bound and unbound Fura-2 can be determined, so that the concentration of free Ca^{2+} can be determined by Grynkiew icz formula (North Broadway; Liang, 2014; Li et al., 1991; Grynkiewicz et al., 1985; An et al., 2002; Xu et al.).

It has been found that injection of agonist of TRPV4 receptor in the lateral ventricle of rats can cause damage to hippocampal neurons, suggesting that excessive activation of TRPV4 receptor can lead to neuronal excitability inhibition or even damage (Huang and Hu, 2017; Li, 2014). As a calcium channel, TRPV4 can cause increased intracellular calcium ion concentration after excessive activation, which in turn causes calcium overload. Studies have shown that calcium overload can directly affect neuronal activity, resulting in reduced oxygen consumption (Huang and Hu, 2017; Li, 2014). The results showed that intracellular calcium ion concentration increased significantly in acute hypoxic exercise until 8 level, while calcium ion concentration in the exhausted state was significantly lower than that in 8 level. Combining immunohistochemistry and mRNA results of TRPV4 channel, it was found that TRPV4 expression was the highest in the exhausted group for each oxygen concentration environment, while the intracellular calcium ion concentration was significantly or very significantly lower than that in the 8-level group, indicating that intracellular calcium overload may concern excessive activation of the TRPV4 channel.

The results of this study showed that intracellular calcium concentration at 8 level in the normoxic environment was significantly lower than that in the hypoxic 2 environment, and it could not be said that the peak value of calcium ion concentration in the normoxic environment was lower than that in the hypoxic 2 environment. It is because according to the study II analyzing exercise capacity of rats, rats can exercise to 10 level in normoxic environment, but can only exercise to 9 level in the hypoxic 2 environment. Therefore, intracellular calcium ion concentration in rat prefrontal cortex may not have reached a peak at 8 level in the normoxic environment.

For many years, calcium ion has been recognized as one major ion that affects important functions of the nervous system (Kawamoto et al., 2012; Rosenberg and Spitzer, 2011; Wang, 2016). Calcium overload is a common mechanism by which many external factors cause excitatory inhibition or even death of nerve cells. Studies on in vitro cultured neurons found that glutamate and other excitatory amino acids could induce neuronal excitability inhibition and even death by mediating calcium ions. Studies have also shown that calcium ion influx causes the membrane channel transition pores to open, producing a large number of free radicals, which in turn causes mitochondria damage and allows H^+ to enter the mitochondria through the membrane channel transition pore. In normal cases, the mitochondrial membrane is transparent to cations, and H^+ should enter the mitochondria through F_1F_0-ATP_{ase} and produce ATP. As the membrane channel transition pore consumes a large amount of H^+, the mitochondrial oxidative phosphorylation process leads to a decrease in ATP synthesis due to the lack of H^+, triggering neuronal excitability inhibition (Liu, 2002). Other studies have shown that significantly increased concentration of free calcium ions in the cytoplasm of nerve cells will cause excessively increased activity of a variety of calcium-dependent protein kinases (such as calcium-activated neutral protease (calpain), caspase, lipase and nucleases), leading to damage or even death of neurons (Chen, 2015; Bano and Nicotera, 2007). Therefore, calcium overload caused by increased intracellular calcium ion concentration can cause neuronal excitability inhibition or even death through various channels.

5. Conclusion

In this study, it was found through living exercise laboratory that cerebral ischemia and hypoxia caused by acute hypoxic exercise can lead to earlier appearance of prefrontal cortical cell edema,

venous congestion than that in normoxic environment and 5,6-EET increase, which may be an important reason for the increased expression of TRPV4 in prefrontal cortex.

The increased expression level of TRPV4 channel activates calcium channel, causing intracellular calcium ion concentration to be overloaded and inhibiting excitability of nerve cells, which leads to decreased brain function and declined exercise capacity accordingly.

This conclusion further explains one important mechanism for athletes' declined exercise capacity in the early stage of altitude training, and provides a new nutritional improvement target for it.

Acknowledgement

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