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Original Article

Exercise Regulates the Lactate Receptor HCAR1 and ERK1/2-PI3K/Akt Pathways to Promote Cerebral Angiogenesis

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Abstract

Background: We aimed to explore the ole and mechanism of lactate receptor (HCAR1) in the angiogenesis of leptomeningeal fibroblast-like cells.

Methods: Human brain fibroblast-like cells were selected and some cells were deactivated, analyzed and compared with HCAR1 mRNA and protein expressions in deactivated/normal cells. HCAR1$^{-/-}$ mice and wild type (WT) mice were selected and divided into WT, WT exercise, HCAE1 KO and HCAE1 KO exercise groups, with 10 mice for each group. HCAR1mRNA and expression levels of proteins in fibroblast-like cells, mRNA and expression levels of proteins in Collagen IV, phosphatidylinositol trihydroxykinase (PI3K), serine threonine kinase (AKT) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) in hippocampus were compared, and the microvessel density (MVD) and diameter were calculated.

Results: mRNA and expression levels of proteins in Collagen IV, PI3K, AKT, ERK1/2 and MVD in hippocampus were significantly higher in the WT exercise group than those in the WT group, microvessel diameter was significantly lower than that in the WT group (P<0.05). mRNA and expression levels of proteins in Collagen IV, PI3K, AKT, ERK1/2 and MVD in hippocampus in the HCAR1 KO and HCAR1 KO exercise groups were significantly lower than those in the WT group, microvessel diameter was higher than that in the WT group (P<0.05). Compared with the HCAR1 KO exercise group, the changes of mRNA in Collagen IV, PI3K, AKT, ERK1/2 and microvascular were not significant.

Conclusion: Exercise can promote cerebral angiogenesis through the activation of the lactate receptor HCAR1 and the ERK1/2-PI3K/Akt signaling pathways.

Keywords: HCAR1 protein; Fibroblast-like cells; Angiogenesis

Introduction

The physiological role of lactate has been controversial after its discovery in biological tissues. Lactate is only an anaerobic metabolic waste, and with the deepening of its research, it can participate in the regulation of intracellular environ-

mental homeostasis, and can also act as a signaling molecule (1,2).

In recent years, lactate can be involved in the regulation of physiological cerebral functions and exert brain protective effects. For example, Roosterman et al (3) found that if the content of lactate in brain increases, the content of insulin-like growth factor, brain-derived neurotrophic factor, vascular endothelial growth factor (VEGF) will also increase, but the specific mechanism has not been clarified. Hydroxy-carboxylic acid receptor 1 (HCAR1), as a lactate-specific receptor, can be expressed in a variety of tissues and organs, such as adipose tissue, brain, gastrointestinal tract, etc. (4). In addition, HCRA1 expression significantly increases in tumor cells, indicating that it can participate in the regulation of tumor cells' growth and development (5).

HCAR1 plays an important role in the conduction of lactate and brain signaling, which can bind to lactate to reduce the spontaneous calcium peak frequency in brain, and is beneficial to blocking neuronal network activity (6,7). HCAR1 can bind to lactate to play a neuroprotective role, inducing VEGFA secretion and promoting cerebrovascular production, which plays a significant role in the repair of brain injury (8). By establishing the middle cerebral artery occlusion model, HCAR1 expression could be significantly up-regulated in both hippocampus and cerebral cortex, which could effectively improve the cerebral infarction area of middle cerebral artery occlusion model and improve the neuronal cell survival rate after the intervention of D-lactate and 3,5-DHBA (9). With the large number of brain fibroblasts-like cells, their functional status changes and their morphological structure changes (10). HCAR1 is highly enriched in leptomeningeal fibroblasts-like cells, indicating that HCAR1 may be a target for promoting cerebrovascular formation and enhancing brain function.

However, the role of HCAR1 in fibroblast-like cells in the leptomeninges has not been fully elucidated, so we aimed to explore it to provide a theoretical basis for the clinical prevention and treatment of brain injury or neurodegenerative diseases.

Materials and Methods

Cell strain

Human brain fibroblast-like cells (Lot No.: HY-iCell-n004, from Beijing Vital River Laboratory Animal Technology Co., Ltd.) were taken for conventional culture and passaged at a ratio of 1:3 according to their proliferation rate to be studied in the logarithmic growth period.

Experimental animals and grouping

A total of 20 2-month HCAR1$^{+/-}$ mice (Lot No.: SCXK (J) 2021-0006, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.) were fed in the same cage at a ratio between males and females of 1:1.

This study was approved by the Animal Ethical Care Committee of Qiqihar Medical University (no. QMU-AECC-2021-225). The 1-month progenies were selected for gene phenotype identification. When the progenies were 2 months old, 20 HCAR1$^{-/-}$ mice and 20 WT mice weighed 18-20g were selected and fed in the SPF laboratory, with the temperature of (22±2) ℃, the humidity of (55±5) %, and the replacement of day and night every 12h (Lighting event 08:30-20:30).

Mice were divided into WT, WT exercise, HCAE1 KO, and HCAE1 KO exercise groups, with 10 mice for each group.

Methods

Inactivation treatment of human brain fibroblast-like cells

Human brain fibroblast-like cells were taken and placed in the 65℃ water and killed in the ice water after 1min, while equal amount of live human brain fibroblast-like cells were added as positive controls.

Treatment of highly intense exercise mice

Mice in WT exercise and HCAE1 KO exercise groups were trained with high-intensity exercise, placed on the treadmill and exercised at uniform speed, gradually accelerated to 14 m per minute

during the first 3min, and then continued exercise at that speed for 1h, 1 time daily for 5w. Mice in the WT and HCAE1 KO groups did not do any exercise. After 5w, each group of mice were anesthetized with isoflurane and killed with hippocampus taken along with part of tissues taken for isolation of fibroblast-like cells.

RT-PCR for expression of relevant factors in cells/tissues

mRNA expression of HCAR1, Collagen IV, PI3K, AKT and ERK1/2 were detected by RT-PC. Total RNA was extracted from human brain fibroblast-like cells or mouse hippocampus by the TRIzol reagent. Primer sequences were designed and reverse transcribed by cDNA by the reverse recording kit, corresponding gene transcription was detected by RT-PCR with β-actin as the internal reference and the relative expression was calculated with $2^{-\triangle\triangle Ct}$.

Western blot for expression of related factors in cells/tissues

The expression of proteins in HCAR1, Collagen IV, PI3K, AKT and ERK1/2 was determined by Western blot. Human fibroblast-like cells or lysates for mouse hippocampus were collected and centrifuged at 12,000 RPM at 4 ℃ for 10 min. The concentration of proteins was determined by the BCA protein assay kit. Electrophoresis was performed according to the time determined by the protein marker. The protein was transferred to PVDF membrane, which was placed in 5% skim milk for sealing. After incubation with primary and secondary antibodies, the membrane was washed, and ECL was added for the film's exposure, development and fixing, and the expression levels of target proteins in HCAR1, Collagen IV, PI3K, AKT and ERK1/2 were analyzed.

Calculation of microvessel density (MVD) and microvessel diameter in mouse brain

Sections of mouse hippocampus were prepared, with the thickness of 4~5μm and images on sections of mice in WT, WT exercise, HCAE1 KO and HCAE1 KO exercise groups were collected by the high-resolution fluorescence image system and processed with the image processing software, such as Zen Lite Blue software. The SimpleGrid Plug-in for Image J system was selected to calculate the number and diameter of microvessels per 1mm2 according to the Delesse principle.

Statistical Methods

Data analysis was performed with SPSS 22.0 software (IBM Corp., Armonk, NY, USA), measurement data with ($\bar{x} \pm s$), ANOVA for multiple group comparisons and *LSD-t* test for pairwise comparisons. $P<0.05$ was considered as a statistically significant difference.

Results

HCAR1 expression was found in both normal and inactive brain fibroblast-like cells, and both HCAR1 mRNA and protein expressions were significantly higher in normal brain fibroblasts-like cells than those in inactive brain fibroblast-like cells (P<0.05, Fig. 1ABC). HCAR1 expression was not found in the HCAR1 KO group, but was found in the WT group ($P<0.01$, Fig. 1DEF).

mRNA expressions of PI3K, AKT, ERK1 and ERK1/2 in hippocampus of mice in the HCAR1 KO group were (0.75±0.05), (0.68±0.08) and (0.55±0.04), respectively, which were (1.67±0.12), (1.49±0.20) and (1.84±0.17) in hippocampus of mice in the WT group. mRNA expressions of PI3K, AKT, ERK1 and ERK1/2 in hippocampus of mice in the HCAR1 KO group were significantly lower than those in the WT group ($P<0.05$). The protein expressions of PI3K, AKT and ERK1/2 in hippocampus of mice in the HCAR1 KO group were (0.78±0.06), (0.65±0.06) and (0.51±0.03), respectively, which were (1.68±0.12), (1.45±0.09) and (1.78±0.12) in hippocampus of mice in the WT group. Protein expressions of PI3K, AKT and ERK1/2 in hippocampus of mice in the HCAR1 KO group were significantly lower than those in the WT group (P<0.01), as shown in Fig. 2 and 3.

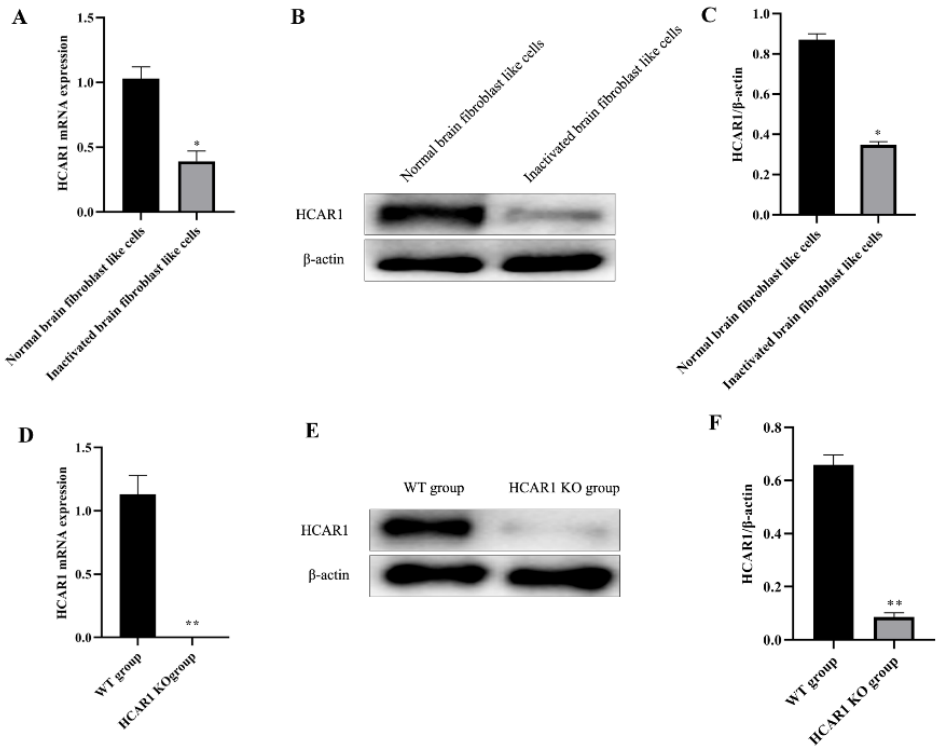

Fig. 1: Analysis on HCAR1 expression in different brain fibroblast-like cells
Note: A: mRNA expression of HCAR1 in human normal/inactivated brain fibroblast-like cells (RT-PCR); B~C: Protein expression of HCAR1 in human normal/inactivated brain fibroblast-like cells (Western blot); D: mRNA expression of HCAR1 in the brain fibroblast-like cells of WT and HCAR1 KO mice (RT-PCR); E~F: Protein expression of HCAR1 in brain fibroblast-like cells of WT and HCAR1 KO mice; a: Normal brain fibroblast-like cells; b: Inactivating brain fibroblast-like cells.* represents P<0.05 vs normal human brain fibroblast-like cells; ** represents P<0.01 vs WT group

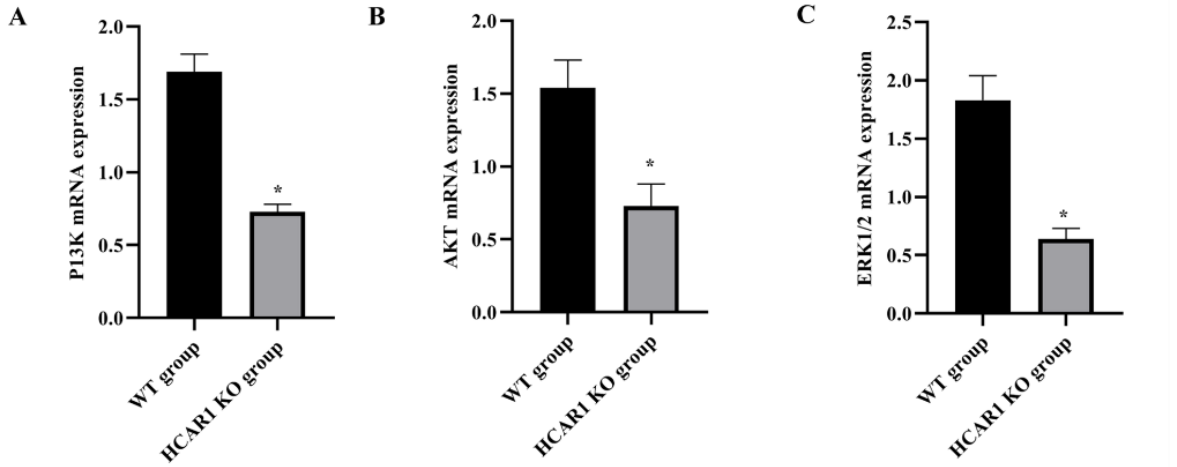

Fig. 2: Comparison of mRNA expression of PI3K, AKT and ERK1/2 between the two groups (RT-PCR)
Note:* represents *P* <0.05 vs. WT group

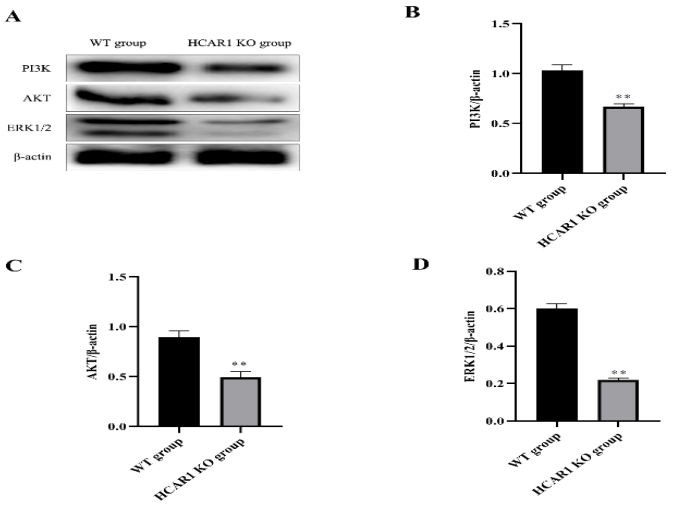

Fig. 3: Comparison of proteins expression of PI3K, AKT and ERK1/2 between the two groups (Western blot)
Note:* represents *P*<0.05 vs WT group; ** represents *P*<0.01 vs WT group

mRNA and protein expressions of Collagen IV, PI3K, AKT and ERK1/2 in hippocampus of mice in the WT exercise group were significantly higher than those in the WT group (*P*<0.05); mRNA and protein expressions of Collagen IV, PI3K, AKT and ERK1/2 in hippocampus of mice in the HCAR1 KO and HCAR1 KO exercise groups were significantly lower than those in the WT group (P<0.01). However, there was no statistically significant difference in mRNA and protein expressions of Collagen IV, PI3K, AKT and ERK1/2 in hippocampus of mice of HCAR1 KO and HCAR1 KO exercise groups (P>0.05), as shown Fig. 4 and 5.

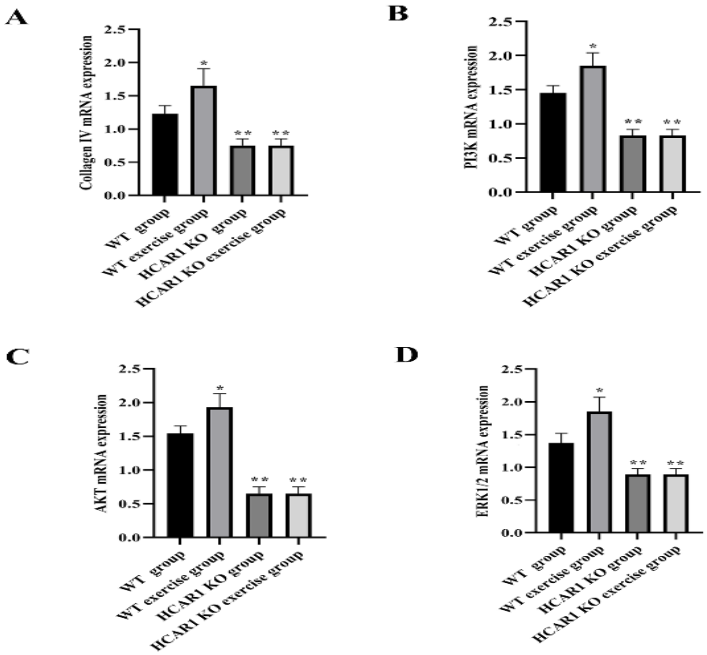

Fig. 4: Comparison of expression of Collagen IV, PI3K, AKT and ERK1/2 all the groups (RT-PCR)
Note: * represents *P*<0.05 vs WT group; ** represents *P*<0.01 vs WT group

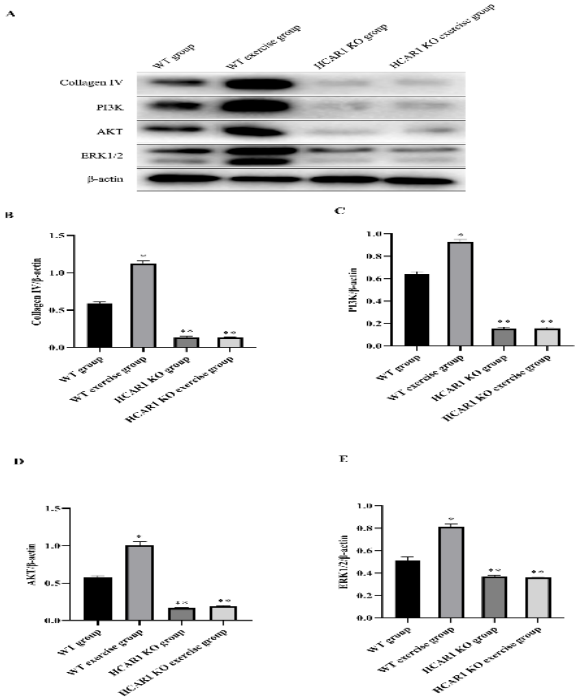

Fig. 5: Comparison of expression of Collagen IV, PI3K, AKT and ERK1/2 of all the groups (Western blot)
Note: A: WT group; B: WT exercise group; C: HCAR1 KO group; D: HCAR1 KO exercise group; * represents $P<0.05$ vs WT group; ** represents $P<0.01$ vs WT group

MVD in the WT exercise group was significantly higher than that in the WT group, with the microvessel diameter significantly lower than that in the WT group ($P<0.05$). MVDs in the HCAR1 KO and HCAR1 KO exercise groups were significantly lower than that in the WT group, with the microvessel diameter significantly higher than that in the WT group ($P<0.05$). There was no statistically significant difference in MVD and microvessel diameter between HCAR1 KO and HCAR1 KO exercise groups, as shown Fig. 6.

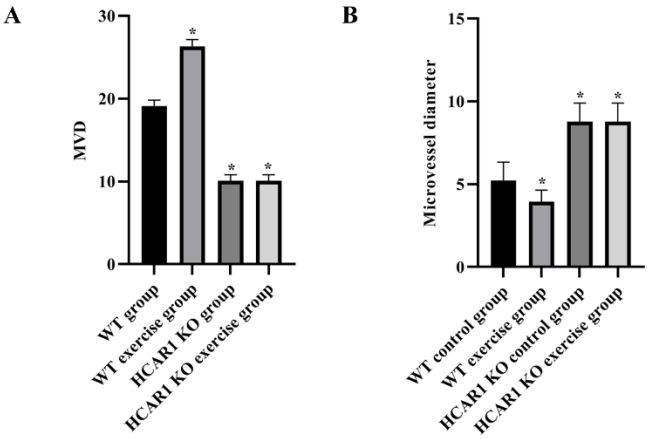

Fig. 6: Comparison of MVD and microvessel diameters of all the groups
Note: A: MVD; B: Microvessel diameter; * represents P<0.05 vs WT control group

Discussion

Lactate is one of the energy sources of the central nervous system, which was not only as a metabolic fuel and buffer, but also as a signaling molecule involved in many pathophysiological links (11,12). HCAR1 plays an important role in regulating the energy metabolism of brain and blood flow in it (13,14). In the central nervous system, mRNA and proteins in HCAR1 are mainly expressed in hippocampus, neocortex and cerebellum. In addition, lactate signals transmitted by specific receptor proteins in the brain was found in the same study, with a preliminary analysis on lactate receptors step by step (15). We aimed to explore the role and mechanism of HCAR1 in the angiogenesis of leptomeningeal fibroblast-like cells, to provide theoretical support for the clinical prevention and treatment of brain injury or neurodegenerative diseases.

HCAR1 is enriched in fibroblast-like cells in leptomeninges, indicating that brain fibroblast-like cells may become target sites to induce cerebro angiogenesis and improve cerebral functions, and HCAR1 may be an important target to induce cerebro angiogenesis (16). To further clarify HCAR1 expression in human brain fibroblast-like cells, we inactivated cells and detected HCAR1 expression in normal human brain fibroblast-like cells with RT-PCR and Western blot, respectively, which showed that mRNA and protein expressions of HCAR1 in normal brain fibroblast-like cells were significantly higher than those in inactivated brain fibroblast-like cells, suggesting that HCAR1 expressed both in normal and inactive brain fibroblast-like cells.

The lactate/HCAR1 signaling systems can significantly attenuate neuronal excitability, and its mechanism may be associated with lactate activation of HCAR1 and AC/cAMP/PKA pathways (17). Lactate/HCAR1 signaling systems can play a neuroprotective role. It blocks the production of NLRP3 and the secretion of IL-1 in microglia, and can play a significant role in improving the neuroinflammatory response (18). Besides, lactate/HCAR1 signaling systems can activate ERK1/2 and AKT pathways, which is beneficial to induce VEGF expression and angiogenesis in hippocampus (19,20). Lactate/HCAR1 signaling systems can mediate different pathways to play neuroprotective and proangiogenic effects, and play an important role in the onset and evolution of neurological diseases.

PI3K/AKT and ERK1/2 signaling pathways are important signaling systems in normal cells. Among them, PI3K/AKT is a common pathway for many membrane receptor signaling to intracellular cells. As one of the downstream effector molecules of PI3K, AKT is the key to this pathway, which can participate in cell biological behavior by regulating relevant proteins downstream of the pathway (21,22). ERK1/2 signaling pathway can be involved in the regulation of cell biological behavior and be activated by many extracellular signals, such as cytokines, hormones and neurotransmitters, but it has not been fully revealed whether targeting this pathway plays a role in brain injury or neurodegenerative diseases (23,24). In this study, we obtained HCAR1 knock-out mice and WT mice, took the hippocampus in mice and isolated the brain fibroblast-like cells, and then detected HCAR1 expression in the cells. It showed that no HCAR1 expression was found in brain fibroblast-like cells of HCAR1 knock-out mice, while HCAR1 expression existed in the WT mouse cells. In addition, we also tested the relevant indicators in hippocampus of mice, showing that mRNA and protein expressions of PI3K, AKT and ERK1/2 in the HCAR1 KO group were significantly lower than those in the WT group, which suggests that knocking down *HCAR1 gene* can block the activation of ERK1/2-PI3K/Akt signaling pathways.

Repeated exercise can increase the content of lactate in brain to some extent, which is conducive to enhancing the cognitive ability (25). During the acute stress, elevated epinephrine can induce increased intracellular cAMP levels and promote increased cognitive function, however, under a chronic stress, a chronic increase of cAMP content can cause cognitive dysfunction (26). With increasing age, cAMP levels in human frontal cortices also increase, which then causes

decreased cognitive function. At this time, repeated physical exercise can promote HCAR1 activation, and then improve the injury caused by the chronic increase in cAMP (27). Lactate is more active in skeletal muscle, and high intensity exercise can promote lactate accumulation in skeletal muscle in the blood, which is similar to lactate injection in brain. It can lead to the up-regulation of VEGFA expression in brain, thus induce neovascularization, which is beneficial to improving neurogenesis and synaptic functions, but the molecular signals related to the up-regulation of VEGFA expression in brain induced by high-intensity exercise have not been fully clarified (28). HCAR1 could be significantly activated by exercise and enhance the proangiogenic effect of lactate. Therefore, this study also used exercise mode to activate HCAR1, which was divided into WT group, WT exercise group, HCAE1 KO group and HCAE1 KO exercise group according to the mice's intensity of exercise, aiming at exploring the role and mechanism of exercise and HCAR1 and PI3K/AKT/ERK1/2 signaling in vasculature formation. RT-PCR and Western blot showed that Collagen IV, C mRNA and protein expressions in hippocampus of WT mice after exercise intervention were significantly higher than those of WT mice without exercise. After knocking down of HCAR1, mRNA and protein expressions of the above indicators in hippocampus of mice were significantly lower than those of WT mice without exercise regardless of exercise intervention. MVD of angiogenesis can directly and clearly indicate the number of neovascular vessels and increased MVD, indicating that the degree of brain injury repair is ideal (29,30).

In this study, we found that MVD in WT mice after exercise intervention was significantly higher than that in WT mice without exercise, and microvessel diameter was significantly lower than that in WT mice without exercise. After knocking out HCAR1 in mice, regardless of exercise intervention, MVD in hippocampus of mice was significantly lower than that in WT mice without exercise, and microvessel diameter was significantly higher than that in WT mice without exercise. It suggests that exercise may promote microangiogenic capillary angiogenesis in brain tissues by activating HCAR1 and ERK1/2-PI3K/Akt signaling pathways. After knocking out *HCAR1 gene* followed by high-intensity exercise, ERK1/2-PI3K/Akt signaling pathways in hippocampus of mice were activated and inhibited, and the expression of Collagen IV, PI3K, AKT and ERK1/2 decreased, and angiogenesis was blocked.

Conclusion

Exercise can promote microangiogenic angiogenesis in brain tissues. The mechanism may be realized by activating HCAR1 and ERK1/2-PI3K/Akt signaling pathways, to provide an experimental basis for mechanical research on brain injury or neurodegenerative diseases and new ideas for the treatment of cerebrovascular diseases.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Competing interest

The authors declare that they have no competing interest.

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