Novel Mechanism of Lowering Blood Pressure by Electroacupuncture: MicroRNA9/P2RX7

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Research Article

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Abstract

Background Our previous studies have reported electroacupuncture(EA) can regulate the relevant indicators of essential hypertension(EH) such as angiotensinII(ANGII),ANG(1-7) and inflammatory cytokines.But the mechanism of EA remains unclear. The goal of this study was to verify P2RX7 as one direct target of miRNA-9,which triggers the release of ANGII and PICs(TNF- α , IL-6)in the PVN.The system of miRNA-9/P2RX7 might be a novel mechanism of lowering blood pressure.

Method Spontaneously Hypertensive Rats(SHRs) were selected as an animal model of hypertension while WKY rats were considered as a blank group.Bipolar cannula implantation were used to inject inhibitors in the paraventricular nucleus(PVN).We measured the systolic blood pressure(SBP) of animals on different days of acupuncture therapy(0,3,7,10,14).We examined the level of microRNA-9 by RealTime-PCR.RT-PCR and western blot was utilized to detect the mRNA and protein levels of P2RX7 in each group.The activation of microglia was detected by immunohistochemistry staining with antibody Iba1.ANGII,IL-6 and TNF- α were detected by ELISA in the PVN and plasma.

Results Our results showed that EA reduces systolic blood pressure of SHR.SHRs.EA increased the level of miR-9 notably comparing to sham groups.In addition,SHRs in the EA group had significantly lower P2RX7 protein and mRNA levels than those in the sham group,while miR9-inhibitor downregulated the expression of P2RX7 in the PVN comparing to EA groups. Immunohistochemistry staining showed that EA treatment and P2RX7 inhibitor significantly inhibited the activation of microglia.We also found that the combination of P2RX7-inhibitor and EA had a better anti-inflammatory effect and better inhibition of ANGII,while using miR9-inhibitor prevented the effect.

Conclusion Taken together, our data demonstrates that P2RX7 is a direct downstream receptor of miRNA-9.Regulating system, miRNA-9/P2XR7, decreases the release of ANGII and alleviates inflammation in the PVN and plasma of SHR,which indicates a novel microRNA-mediated mechanism of EA.Furthermore, the present investigation provides the combination treatment of P2RX7-inhibitor and EA as a better intervention in the area of anti-inflammatory.

Background

It is generally accepted that angiotensinII and inflammation might contribute to essential hypertension(EH), which is a major risk factor for cardiovascular disease with a large waste of medical and social resources. Our previous studies have reported electroacupuncture(EA) can regulate the relevant indicators of EH such as ANGII,ANG(1-7) and inflammatory cytokines.Electroacupuncture has become a hot spot of nonpharmaceutical intervention on lowering blood pressure especially in China,while its molecular mechanism remain poorly understood.Recently,much of evidence revealed the crucial role of microRNA in the whole process of blood pressure regulation.Thus,we aimed to find a novel microRNA-mediated mechanism through electroacupuncture treatment in Spontaneously Hypertensive Rats(SHRs).

Introduction

MicroRNAs control the basic functions of almost all cells related to the cardiovascular system and mediate the regulated targets and signaling pathways, such as Angiotensin II^[1,2](AngII), pro-inflammatory cytokines(PIC) in the paraventricular nucleus^[3,4] and purinergic receptor P2RX7^[5,6]. MiRNA-9, one of the most ancient and adequate microRNAs existing in our neural network, promotes the development of the nervous system^[7]; further study demonstrated that miRNA-9 is considered as a key regulator of the inflammatory response in activating microglia through inhibiting the transcription of NF- κ B^[8]. Elevation of the inflammatory response reactant involving tumor necrosis factor (TNF) and interleukin-1(IL-1) in Paraventricular nucleus(PVN) of hypothalamus will predispose individuals to higher blood pressure^[9]. Moreover, a significant increase of miRNA9 was observed by electroacupuncture in a rat model of middle cerebral artery occlusion (MCAO)^[10]. It has been proved that downregulation of microRNA-9 increases matrix metalloproteinase-13 expression levels and facilitates osteoarthritis onset^[11]. Evidence also reveals the inhibitory roles of miRNA9 on papillary thyroid cancer through targeting BRAF pathway^[12]. EA is an effective alternative therapy for the treatment of hypertension. Although the therapeutic relationship between microRNA and EA still remains unclear, there is increasing evidence supporting microRNA might be an essential part of the acupuncture therapeutic mechanisms^[13,14,15].

Recent studies demonstrated that there is an interaction between AngII and inflammatory cytokines in paraventricular nucleus of the hypothalamus, which give rise to a variety of cardiovascular diseases^[16-18]. Significantly, the release of AngII and PIC during the neuroinflammation stimulates PVN through the purinergic receptor P2RX7 in microglia.

As an ATP-gated nonselective cation channel, it was confirmed that P2RX7 leads to an increase of inflammatory cytokines^[19]. In addition, activation of P2RX7 and excess renal interstitial angiotensin jointly led to the secretion of interleukins and growth factors which contribute to the renal injury caused by hypertension^[20]. Increased expression of the P2RX7 was detected in hypertensive rats induced by a high-salt diet; nevertheless, animals exhibited reduced blood pressure after knocking out P2RX7 gene^[21]. Evidence shows that P2RX7 plays a crucial role in the area of acupuncture therapy, including traumatic brain injury(TBI) and acid- and purine-induced pain^[22-23]. It has not been fully elucidated, however, whether this pathway influences the treatment of hypertension by EA.

Despite the promising results obtained from increasing factors such as ANGII and IL-1 β ^[9,19,20], most of the researches merely targeted on a single ion channel or a single type of microRNAs. Multiple investigators reported that microRNAs modulate the purinergic P2RX7 signaling network. As an example, P2RX7 are regulated by microRNA-216b in human breast cancer^[24]. Similar regulatory has been found in the experimental rat model of epilepsy and lung surfactant secretion^[25-26]. So we harbor the idea that there is a more integrated mechanism which combines both of them and the downstream targets of microRNAs. In the present study, We hypothesized that regulating system—miRNA-9/P2RX7 participates in microglial activation of SHR and keeps blood pressure from rising by EA. We used the gene detection and

molecular biologic analysis to verify P2RX7 as one direct target of miRNA-9 and we further examined the release of ANGII and PICs(TNF- α , IL-6)to validate the inhibition of P2RX7.

Materials And Methods

1. Experimental animals

Twelve-week-old male spontaneously hypertensive rats and Wistar Kyoto rats(250-280g) were obtained from Beijing Charles River Experimental Animal Technology Co., Ltd.,(Beijing, China). The license number was SYXK (Beijing)2016-0006.All animals were kept in solitary cages with a constant temperature of 25-27°C, a 12-hour light/dark cycle, and free access to water and food. The rats were fasted from food and water on the day before the tissue extraction.Experiments were carried out after receiving approval from the Beijing University of Chinese Medicine's Institutional Animal Care and Use Committee.All animal surgeries we performed were under the circumstance of 10% chloral hydrate anesthesia strictly to minimize suffering.

2. Experimental design

SHRs were divided among six groups at random:sham+cerebrospinal fluid(CSF), miR9-inhibitor+EA, P2RX7-inhibitor,P2RX7-inhibitor+EA,EA,non-stimulated control(n=10 rats per group) while WKY rats were considered as a blank comparison.All groups with EA were used for observing the effect of acupuncture treatment on regulating the expressions of miR-9 and P2RX7 pathways.The relevance of P2RX7 in acupuncture's therapeutic effects was investigated by using P2RX7-antagonist and EA groups.Research plan of surgery and blood pressure measurement is shown in Fig.1a.

3.Bipolar cannula implantation in the paraventricular nucleus

We implanted bipolar cannulas in PVN of the animals divided in inhibitor and sham groups to inject inhibitors of miRNA-9 and P2RX7.Chloral hydrate (10%,0.35 ml/100g) was used to anesthetize rat intraperitoneally.After cutting epicranium with surgical scissors,the rats were fixed in the stereotaxic apparatus in prone position.Then we mark the position of paraventricular nucleus(1.8mm behind bregma, 0.4mm on both side of sagittal suture, 7.9mm under calvarium)through the stereotaxic apparatus under the guidance of stereotaxic atlas of rat brain and previous study^[27-28].Operational drills were carried out lightly in the both side of PVN after disinfection.Next,we implanted the cannulas into fixed orifice and applied the dental cement onto operative incision.After setting of the cement,we screwed the lid of bipolar cannulas. The procedure was performed as gently as possible to minimize the animal's agony, and an electric blanket was utilized to keep the rats warm.Surgical sketch is shown in Fig.1b.

4. Intraventricular Injection

Two antagonist: A438079(P2RX7 antagonist,hydrochloride hydrate, 10 μ mol) and antagomir-9(mir-9 antagonist, 10 μ mol)have been injected into PVN by microsyringe. The needle of microsyringe connected

with a suited PE pipe was inserted into both sides of the bipolar cannula after air exhaust. The dosage of PVN on one side was 100nL each time and the injection lasted for about 5min. Injection was administered at 12-hour intervals. Detailed reagents are listed in Supplemental Table 1.

5. Electroacupuncture treatment

Electroacupuncture treatment was performed once a day for two weeks, 5 days after the surgery. After disinfecting with 75% alcohol, sterilized disposable steel needles (0.16mm×7mm, Zhong Yan Tai He Co., Beijing, China) were inserted perpendicularly into two acupoints—bilateral Quchi (LI11) and Taichong (LR3) at a depth of 4-5 mm. Figure 1(c) depicts the precise location of acupoints. Pair of needles on the same acupoints were connected with the output terminals of an EA apparatus. Sparse (2Hz) and dense (10Hz) pulse alternation (2.5s interval) was given in the whole treatment so that we might be able to keep animals from becoming accustomed to the same stimulated frequency of EA. Lighter intensity (1mA) was applied to keep SHR from grumpy and anxious. To allay their fears, we made a mini-acupuncture room with opaque socks during the treatment. Needles were removed after a Twenty-minute EA stimulation. Catching-grasping was performed in the sham and non-stimulated control groups without acupuncture treatment, and the animals were put into socks for twenty minutes either.

6. Methods

6.1 Blood Pressure Measurement

All rats were preheated by thermostat to adjust the constant temperature at 36°C for 15 minutes. Non-invasive blood pressure apparatus was used to measure the tail pressure of rats in a quiet and awake state for 3 consecutive times. Rats were fixed by meshed nets and appropriate sets of tail manometer. The mean value was taken to avoid the influence of noise and other external environmental stimuli on systolic blood pressure (SBP). SBP were measured from 8:00 a.m. to 12:00 a.m. on day 0 (1d before acupuncture) and on days 3, 7, 10, and 14 of acupuncture to reduce errors. The whole process of capture were gentle to prevent provoking the rats, so as not to cause fluctuations.

6.2 RNA extraction and Real-time QPCR

Total RNA containing miRNAs and mRNAs was extracted from Paraventricular nucleus of the hypothalamus by TRIzol reagent (Invitrogen, America, 10296028) according to the manufacturer's instructions. And the mixture was incubated for 50 minutes at 50 degrees Celsius, then for 5 minutes at 85 degrees Celsius, before being cooled on ice. CDNA Synthesis Kit (ABI-Invitrogen, 11752050) and SYBY-qPCR mix (ABI-Invitrogen, 4472920) were used following the manufacturer's protocols. Endogenous control (U6) was considered as an internal reference. Melting curves were used to estimate the purity of the amplified band. Relative quantification was performed by determining the n-fold differential expression using the $2^{-\Delta\Delta Ct}$ method. The amplification melt peak of PCR and the sequences of primers for miR-9, P2RX7, GAPDH are listed in Supplemental Table 2.

6.3 ELISA

Supernatants from hypothalamus homogenates and plasma taken from the abdominal aorta were used for detecting the concentrations of ANGII, IL-6, and TNF- α with corresponding ELISA kits by using Rayto RT-6100 according to the manufacturer instructions.

6.4 Western Blot

In brief, the Pierce BCA Protein Assay kit (Servicebio G2026) was used to assess the protein concentrations in the PVN according to the manufacturer's instructions. A total of 20 μ g of protein was supplied each lane and a polypropylene gel with a concentration of 10% was created. 10 percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE Servicebio G2003) was used to separate the samples, which were then electronically transferred onto PVDF membranes (0.45 μ m Servicebio G6015). 300mA constant flow membrane was used for half an hour. Membranes were put into the incubation tank equipped with TBST, which were blocked by skim milk at room temperature for 30 min. After Primary antibody (P2RX7, β -actin) added, membranes were incubated at 4 $^{\circ}$ C overnight. The secondary antibodies were incubated at room temperature for 90 min and AlphaEase FC software was used to analyze the protein expression. Detailed reagent are listed in supplemental table 3.

6.5 Immunohistochemistry and Image Analysis

Hypothalamus tissues were collected after the treatment of EA, which were immersed in 4% paraformaldehyde for 24 hours. Samples containing PVN were sliced to a thickness of 10 μ m with a Leica frozen slicer. The slices were immersed in xylene, anhydrous ethanol, 85% alcohol, 75% alcohol, distilled water successively. The repair box filled with citric acid antigen repair fluid (PH6.0, Pinuofei biological technology co., LTD, Wuhan china) was heated in the microwave oven until boiling, and the tissue sections were placed in the repair box for antigen repair with high fire for 10 minutes. Then slides were immersed in a 3 percent hydrogen peroxide solution and incubated at room temperature for 25 minutes without being exposed to light. After washing by PBS and shaking on a orbital shaker (WD-9405A, Beijing) for 3 times (5min each), a PAP-pen was used to draw a circle around the tissue and 3% BSA (Solarbio A8020) was added to cover the tissue in the circle, which was sealed at room temperature for 30min. After that, PBS was added to the slides with drops of three primary antibody prepared in a certain proportion and slides were placed in a wet box at 4 $^{\circ}$ C and incubated overnight. The secondary antibodies (HRP marked) of the corresponding species in the immunohistochemistry kit were added to cover the tissues, which were incubated at room temperature for 50min. After DAB chromogenic reaction, Harris hematoxylin (Pinuofei biological technology co.) was used for counterstaining about 3min. Finally, microscope (CIC, XSP-C204) and scanner (3DHISTECH KFT) examination were used for image acquisition. Primary and secondary antibody are listed in supplemental table 4. Image J were used to calculate average optical density (AOD) of every picture.

7. Statistic Analysis

All data were analysed via SPSS 26.0 (SPSS Inc., IBM, USA) and presented as mean $\pm$ SD. Data with normal distribution were analysed by one-way analysis of variance (ANOVA) followed by an LSD test and Bonferroni post-test for determining the group differences. Linear regressions were examined by Pearson's correlation analysis. $P < 0.05$ was considered statistically significant.

Results

1. EA reduces systolic blood pressure of SHRs

We measured the systolic blood pressure (SBP) of animals on different days of acupuncture therapy (0, 3, 7, 10, 14, Fig. 2). There was no significant difference around all SHR groups before surgery and treatment ($P > 0.05$), and there was a prominent increase compared with WKY rats ($P < 0.01$). It showed that SHRs maintained a relatively stable state of hypertension during all experiments. The therapeutic effect of EA began to show after 7 days of treatment ($P < 0.05$). After a 14-day treatment, SHRs in P2RX7-inhibitor+EA and EA group showed clearly reduced compared to sham and miR9-inhibitor +EA group ($P < 0.05$) while no significance was found between P2RX7-inhibitor+EA and EA group ($P > 0.05$). Detailed data of SHRs and WKY rats was shown in supplement table I.

2. P2RX7 is a direct target of miRNA-9 via EA treatment.

To verify the relationship between P2RX7 and miRNA-9 during EA therapy, a series of experimental tests were performed on SHRs. RT-PCR and western blot was utilized to detect the mRNA and protein levels of P2RX7 in each group (Fig. 3a,b). As shown in the picture, the overall levels of P2RX7 were higher in the control group when compared to the WKY rats ($P < 0.001$). SHRs in the EA group, on the other hand, had significantly lower P2RX7 protein and mRNA levels than those in the sham group ($P < 0.01$). Since miR-9 has neuroprotective in our brain, we postulated that the suppression of P2RX7 in SHRs following acupuncture therapy might be due to the modulation of miR-9 expression. So we further examined the relative expression of miR-9 in the PVN of SHRs (Fig. 3c). Our results revealed that miR-9 levels in control groups were significantly decreased in comparison to the level among WKY rats ($P < 0.05$). Furthermore EA increased the level of miR-9 notably comparing to sham groups ($P < 0.001$). As we expected, miR9-inhibitor downregulated the expression of P2RX7 in the PVN comparing to EA groups ($P < 0.01$). To evaluate if miR-9 levels of SHRs influenced P2RX7 expression in the PVN, we analyzed the relationship between miR-9 and P2RX7. The results demonstrated a substantial positive linear connection between miR-9 and P2RX7 protein/mRNA levels in the PVN ($P < 0.01$ and $P < 0.05$, respectively). Therefore, expression of P2RX7 may be favorably regulated by miR-9.

3. MiRNA-9/P2RX7 mediates microglial activation in the PVN of SHRs

Microglia are inherent immune cells in the central nervous system, however, microglial activation was associated with neurotoxicity and neuroinflammation which contributes to hypertension. So we validated whether P2RX7 may have participated in this process. The activation of microglia was detected by immunohistochemistry staining with antibody Iba1 (Fig. 4a). We found that EA treatment and P2RX7

inhibitor significantly inhibited the activation of microglia($P<0.001$),after calculating AOD(integrated optical density/area) in the Fig.4(b).In addition,the pictures showed that miRNA9 inhibitor attenuated the inhibitory process of EA and the value of AOD supported it($P<0.01$,compared to EA group).

4.The combination of P2RX7-inhibitor and EA had a better anti-inflammatory effect,while using miR9-inhibitor prevented the effect.

To examine the anti-inflammatory efficacy of EA and two inhibitors, we measured inflammatory cytokines in the PVN and plasma, including IL-6 and TNF- α .(Fig.5).It was found that EA reduced serum levels of IL-6 and TNF- α ($P<0.001$).In addition, we found miR9-inhibitor abolished the anti-inflammatory potential of EA ($P < 0.01$ and $P < 0.05$, resp.),whereas P2RX7-inhibitor did not prevent this effect.The combination of P2RX7-inhibitor and EA treatment could significantly reverse the overexpression of IL-6 and TNF- α ,compared to EA ($P < 0.01$ and $P < 0.05$) and P2RX7-inhibitor groups ($P < 0.01$ and $P < 0.05$).

5.P2RX7-inhibitor+EA inhibits the release of ANGII,while miR9-inhibitor prevented the effect.

To verify the effect of miR-9 on hypertension,we further examined the release of ANGII in each group(Fig.6 a,b).The results indicated that EA strongly suppressed the level of ANGII in comparison with the sham group ($P<0.001$).Then we found that the inhibitor of miR-9 in PVN increased the secretion of ANGII($P<0.05$), compared with SHR treated by EA, as detected by ELISAs.And as expected,P2RX7-inhibitor+EA group significantly repressed the ANGII,which had a better effect among EA and P2RX7 groups.($P < 0.01$, $P < 0.05$, resp.)Therefore,targeting miR-9/P2RX7 could control inflammatory responses and the release of ANGII after microglial activation.Next we aimed to assess whether PICs and ANGII of SHRs have a mutual effect under the treatment by EA(Fig.6 c,d).Pearson linear correlation analysis showed that the levels of PICs(IL-6 and TNF- α) and ANGII both had a strong correlation respectively($P<0.001$).

Discussion

Our study revealed that EA reduces the systolic blood pressure of SHRs by reducing the levels of inflammatory cytokines and ANGII in PVN and plasma,which were associated with increased expression of microRNA9 and downregulation of its direct target P2RX7.In addition,EA mimics the anti-inflammatory response of P2RX7 antagonist and reverses the overexpression of P2RX7. Furthermore, even though there was no significance in SBP between P2RX7-inhibitor+EA and EA groups,we provide evidence that the combination of P2RX7-inhibitor and EA had a better inhibition of inflammatory cytokines and ANGII,while using miR9-inhibitor prevented the effect.

As a common chronic disease,essential hypertension is characterized by elevated blood pressure resulting from a variety of inducements, which causes about 62 percent of strokes and 49 percent of heart attacks^[29].Renin-angiotensin system(RAS) in PVN is considered as a crucial part in central nervous blood pressure regulation.Angiotensin II, the main product of RAS, plays an important role in the increase of central reactive oxygen species^[30] (ROS), secretion of pro-inflammatory factors^[31](PIC), excitation of

sympathetic nerve^[32] and contraction of arteriolar smooth muscle^[33], which has much significance in the occurrence and development of hypertension. Inflammatory process has been proved to mediate neuronal excitability and even affect neuronal discharge rate, possibly damaging neurohomeostasis of PVN in the hypothalamus^[34]. Inflammatory factors such as TNF- α and IL-6 are significantly increased in PVN due to increased sympathetic nerve activity and cardiac sympathetic afferent reflex, resulting in increased blood pressure^[35]. In this study, we revealed that EA lowered the SBP by reducing the levels of inflammatory cytokines and ANGII in the PVN and plasma. We also found a linear correlation between PICs (TNF- α and IL-6) and ANGII in the PVN of all SHR, which implies that PICs and ANGII may influence each other.

P2X7 receptors are extensively expressed in immune cells, such as dendritic cells, mast cells, and monocytes, as an ATP-gated ion channel^[19]. Studies have shown that activation of P2RX7 accelerates the progression of renal injury induced by angiotensin II^[36] and vascular contraction^[20,37]. Of note, previous studies have already validated P2X7 receptors' activation leading to a strong inflammation response by increasing C-reactive protein (CRP), IL-1 β , IL-6, tumor necrosis factor α , and interferon, significantly increased in the plasma^[38]. Our results support these conclusions. The present study demonstrates the protein and mRNA levels of P2RX7 in the PVN were increased among SHR, which was controlled by EA treatment. Our results also revealed that the increase of serum ANGII, IL-6 and TNF- α in SHR was reversed by blocking P2RX7 through EA or antagonist.

Microglia is an inherent immune cell in the central nervous system, which plays an important role in physiological and pathological processes^[39]. P2RX7 is a cationic channel purine receptor with ATP ligand, which is widely expressed in microglia^[40]. Studies have shown that P2X7 receptor can activate NF- κ B through PI3K/AKT signaling pathway, mediating microglia activation and participate in neuroinflammation^[41]. In our study, immunohistochemistry staining supports the notion that miRNA-9/P2RX7 mediates microglial activation and we found that P2RX7 inhibitor and EA treatment inhibited the activation.

MicroRNAs are considered as new potential molecular biomarkers which guide clinical treatment in cardiovascular diseases by regulating the expression of multiple pathways^[42]. As one of the most ancient microRNAs, it has been proved that miRNA-9 targets numerous receptors such as PDGFR- β , TLK and Toll pathways^[43,44,45], implying that miRNA-9 may be a potential biomarker in hypertension. In this study, we found that the expression of miRNA-9 was increased by EA in SHR. Moreover, recent researches reported that miRNA-9 promotes the development of the nervous system^[7]. Several studies have addressed miRNA-9 brings about a striking neuroprotective effect in our brain^[46,47]. In agreement with these researches, our study revealed a significant positive correlation existed between miRNA-9 and P2RX7 levels. We also found that the increasing level of miRNA-9 significantly alleviated neuroinflammatory disorders and neuroinflammation in SHR, which was mediated by reducing P2RX7 expression.

The clinical treatment of hypertension mainly relies on taking oral drugs, which has several deflections such as bad side effects, poor compliance, and unstable effects^[48].Electroacupuncture is a form of acupuncture which incorporates electrical stimulation and it is widely used in many diseases, including stroke, depression,and insomnia^[49,50].Compared with drug therapy, EA has natural advantages:It not only has significant curative effect, but also has the characteristics of simple operation,environmental protection,lower toxic and side effects, which results in higher compliance of patients.In recent years,studies on mechanism of EA focused on different types of miRNAs and its downstream targets^[51,52].It is believed that the mechanism of EA is a complex biological process involving a variety of pathways and mediators.

In brief,augment of miR-9 through EA treatment inhibits the increase of ANGII and inflammatory responses by means of downregulating P2RX7 expression.System of MiRNA-9/P2RX7 is a novel mechanism of lowering blood pressure by electroacupuncture and the combination therapy of P2RX7-inhibitor and EA might serve as an example for potential medication development or treatment.

Conclusion

Our data demonstrates that P2RX7 is a direct downstream receptor of miRNA-9.Regulating system, miRNA-9/P2XR7, decreases the release of ANGII and alleviates inflammation in the PVN and plasma of SHR,which indicates a novel microRNA-mediated mechanism of EA.Furthermore, the present investigation provides the combination treatment of P2RX7-inhibitor and EA as a better intervention in the area of anti-inflammatory.

Abbreviations

SBP systolic blood pressure

PVN paraventricular nucleus

SHR Spontaneously Hypertensive Rats

EA Electroacupuncture

EH essential hypertension

PIC pro-inflammatory cytokines

AngII Angiotensin II

IL interleukin

TNF tumor necrosis factor

Declarations

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Consent for publication

The author agrees to publication in the Journal of Neuroinflammation.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xin Liu,Bingnan Yue,Jiao Sun,Zixian Zhou and Qingguo Liu conceived and designed the experiments.Yue Bingnan wrote the manuscript. Xiaolu LI modified the manuscript. All authors reviewed the manuscript.

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Figures

(a)

(b)

(c)

Figure 1

a Sketch of EA treatment and blood pressure monitoring, SBP systolic blood pressure. **b** Acupoint locations of Quchi (LI11) and Taichong (LR3). **c** Simplified sample graph of bipolar cannula implantation in paraventricular nucleus.

Figure 2

Evolution of SBP in each group(mean±SD). **P<0.01 all SHR vs. WKY rats; SBP after 7 days treatment, #P<0.05, P2RX7-inhibitor+EA and EA group vs. Sham; SBP after 14 days treatment, ##P<0.01, all EA groups vs. Sham; ▲P<0.05, P2RX7-inhibitor+EA and EA group vs. miR9-inhibitor+EA; NS, there was no significance between P2RX7-inhibitor+EA and EA group.

Figure 3

P2RX7 is a direct target of miRNA-9 via EA treatment. (a) The mRNA expressions of P2RX7 in the PVN of WKY, Sham, EA, MiR9-inhibitor+EA, Control groups were measured using quantitative real-time PCR. (n=5) (b) Using western blot, the protein levels of P2RX7 and its associated β -actin bands in the PVN were measured in WKY, Sham, EA, MiR9-inhibitor+EA, Control (from left to right) groups. (c) After 14 days

following acupuncture therapy, the relative level of miR-9 in the PVN was measured using quantitative real-time PCR.(d)Linear regression,examined by Pearson's correlation analysis,described the relative levels of miR-9 and P2RX7 protein in the PVN(n=25 each correlation).(e)Linear regression described the relative levels of miR-9 and P2RX7 mRNA in the PVN(n=25 each correlation).*P<0.05,**P<0.01,***P<0.001.

Figure 4

MiRNA-9/P2RX7 mediates microglial activation in the PVN of SHRs. **a** Immunohistochemistry staining of microglia in the groups of Sham,EA,MiR9-inhibitor,P2RX7- inhibitor,WKY with Iba1 antibody,40 $\times$ graph bar=20 μ m. **b** Relative quantification of average optical density(AOD integrated optical density/area) of Iba1 staining in PVN. n = 3 in each group,***P<0.001,**P<0.01.

Figure 5

The combination of P2RX7-inhibitor and EA had a better anti-inflammatory effect,while using miR9-inhibitor prevented the effect.**a,c**. Levels of IL-6 in the plasma and PVN were detected in sham control,miR9-inhibitor +EA,P2RX7-inhibitor+EA,EA,WKY,P2RX7-inhibitor group by ELISAs(n=5).(b,d)The levels of TNF- α in the plasma and PVN were detected in sham,control,miR9-inhibitor+EA,P2RX7-inhibitor +EA,EA,WKY,P2RX7- inhibitor group by ELISAs(n=5). *P<0.05, **P<0.01, ***P<0.001,compared as indicated.

Figure 6

P2RX7-inhibitor+EA inhibits the release of ANGII, while miR9-inhibitor prevented the effect. (a, b) The levels of ANGII in the plasma and PVN were detected in sham, miR9-inhibitor+EA, P2RX7-inhibitor+EA, EA, WKY, P2RX7-inhibitor groups after treatment by ELISA ($n=5$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared as indicated. (c, d) The linear correlation between PICs (TNF- α and IL-6) and ANGII in PVN of all SHR by the scatter plot, $n=25$, for each correlation.

Figure 7

EA reduces the systolic blood pressure of SHRs by reducing the levels of inflammatory cytokines and ANGII in PVN and plasma, which were associated with increased expression of microRNA9 and downregulation of its direct target P2RX7.

Supplementary Files

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