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TRPC1 as an ischemic sensation of focal cerebral ischemia 14

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Abstract: Ischemia is a complex disease which is related to oxidative stress, calcium overload, inflammation. The transient receptor potential (TRP) channels are generally described as calcium-permeable cationic channels with activation property of cellular sensation, such as, thermal inductance and stretch. However, its activation to ischemic stress has not been reported. In present study, we try to explore whether TRPC1 expression is induced by ischemia in middle cerebral artery occlusion (MCAO) model in mice and patients with ischemic stroke. We found that the mRNA level of TRPC1 was increased in the mice cortex, peripheral blood of mice after ischemia induced by middle cerebral artery occlusion (MCAO) model for 2 h, as well as in patients with ischemic stroke compared to control by using real-time quantitative PCR. Then we examined the molecules related with ischemic stroke, hypoxia inducible factor-1 α (HIF-1 α), thioredoxin-1 (Trx-1), tumor necrosis factor- α (TNF- α), cyclooxygenase-2 (COX-2). The expressions of these molecules had similar changes. Moreover, we analyzed the relationships between TRPC1 and these molecules expressions in patients with cerebral ischemic stroke by nonparametric Spearman's correlations and step-wise linear regressions analysis. Our results showed that TRPC1 expression was closely related to other molecules expressions. Thus, TRPC1 may be taken as an ischemic sensation of focal cerebral ischemia.

Key words: TRPC1; ischemic stroke; oxidative stress; inflammation

Abbreviations: MCAO Middle cerebral artery occlusion; TRP Transient receptor potential; HIF-1 α Hypoxia inducible factor-1 α ; Trx-1 Thioredoxin-1; TNF- α Tumor necrosis factor- α ; COX-2 Cyclooxygenase-2

Introduction

Ischemic stroke results from cerebral blood flow interruption, which leads to local brain tissue and cellular ischemia and hypoxia. The overload of intracellular Ca²⁺, reactive oxygen species (ROS), and inflammation are related to brain tissue injury after ischemic stroke [1].

Members of the TRP superfamily are expressed in all mammalian organs and cell types. The great progress has been made in elucidating their involvement in health and disease in recent years [2]. The TRPC subfamily was established following the identification and cloning of TRPC1, the first recognized mammalian TRP channel[3]. TRPC1 functions as Ca^{2+} influx channel[4, 5]. TRPC1 has been reported to be involved in Parkinson's Disease[6]. Evidence that TRPC1 also forms a stretch-activated channel was recently presented[7]. Neuronal TRP channels has been reported as thermometers, pathfinders and life-savers[8].

However, the TRPC1 expression in brain tissue and peripheral blood after cerebral ischemia has not been reported. In present study we detected the expressions of TRPC1 and ischemia related molecules in brain and peripheral blood of MCAO model and patients with ischemia stroke.

Material and methods

Tissue collection and the ethics

Thirty patients with ischemic stroke and thirty normal people as control, male and female, aged at 50-70 years old, were recruited for this study. Blood samples were collected before receiving treatment after ischemic stroke 4 h for 2 day. The diagnosis of cerebral infarction was carried out according to the Fourth Conference on the diagnostic criteria of cerebrovascular disease and fMRI. All patients were hospitalized in the First People's Hospital in Yunnan Province, Kunming, China. The ethics committee of the First People's Hospital in Yunnan Province approved this study and all procedures were performed in accordance with the Code of Ethics of the World Medical Association. All the patients and control people agreed to give their blood for biochemical examination. Samples were immediately frozen in liquid nitrogen and stored at -80°C for later biochemical analysis.

Animals

Male C57BL/6 mice (Chongqing Medical University, China), weighing 25-30 g, were used in the experiment. The mice were housed in plastic cages and maintained in 12 h (light)-12 h (dark) cycle and had free access to food and water. Room

1 temperature was maintained at $28 \pm 1^{\circ}\text{C}$. Animal care and all animal procedures were
2 performed in accordance to the National Institutes of Health Guide for the Care and
3 Use of Laboratory Animals and was approved by the local Committee on Animal Use
4 and Protection.
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7 **Transient mouse MCAO model**

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10 Focal cerebral ischemia was induced by middle cerebral artery occlusion
11 (MCAO) using a nylon monofilament suture as described previously[9]. The
12 C57BL/6 mice were anesthetized with pentobarbital sodium (50 mg/kg body mass).
13 MCAO (right carotid artery) was performed according to the method of Longa et
14 al[10]. Briefly after a midline skin incision, the right common carotid artery was
15 exposed. Then a nylon monofilament of length 2 cm was gently advanced from the
16 external carotid artery up to the internal carotid artery for a length of about 10-11 mm,
17 until the filament was at the branch of the internal carotid artery (ICA) and external
18 carotid artery. At this point, the intumescent structure of the monofilament occluded
19 the origin of the middle cerebral artery (MCA) and almost entirely blocked the
20 sources of blood flow from the ICA, anterior cerebral artery, and the posterior
21 cerebral artery. The same surgical procedures were performed on the Sham animals
22 but without occlusion of the MCA. Throughout the procedure, a thermometer was
23 inserted into the rectum of the mice and the body temperature was maintained at $37 \pm$
24 0.5°C with a thermostatically controlled infrared lamp. Two hours later, the mice
25 was cervical dislocated and then get the blood from orbital cavity. Then mice were
26 perfused with PBS prior to tissue extraction.
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45 **qRT-PCR**

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47 Total RNA was extracted from 0.1 g mice cerebral cortex, 200 μl peripheral
48 blood, and 200 μl human peripheral blood using Trizol reagent kit (CWBIO
49 Corporation, China) and converted to cDNA using the RevertAid™ First Strand
50 cDNA Synthesis Kit (Fermentas, Walldorf Baden, Germany). The product was
51 analyzed using a Prism 7300 Sequence Detection System (Applied Biosystems, USA).
52 The following primer pairs were selected for quantitative real-time polymerase chain
53 reaction (qRT-PCR) - mouse β -actin forward: 5'- CAG TTC GCC ATG GAT GAC
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GAT-3', reverse: 5'- ATC TGG GTC ATC TTT TCA CGG TTG-3'; mouse TRPC1 forward: 5'- GCT GAG GAT GAC GTG AGG AG-3', reverse: 5'- GGA CGA CAG AGT GGA GCG -3'; mouse HIF-1 α forward: 5'- CAG CCT AAC AGT CCC AGT GAA-3', reverse: 5'- GTG CTC ATA CTT GGA GGG CT -3'; mouse Trx-1 forward: 5'- CAA AAG GTG GGG GAG TTC T-3', reverse: 5'- TAA TCA GAT GGC AGT TGG GTA T -3'; mouse COX-2 forward: 5'- CAG TCA GGA CTC TGC TCA CG-3', reverse: 5'- ATC CAG TCC GGG TAC AGT CA -3'; mouse TNF- α forward: 5'- GCC TAT GTC TCA GCC TCT TCT C-3', reverse: 5'- TGG GAA CTT CTC ATC CCT TTG G -3'. Human GAPDH forward: 5'-CAA GGC TGA GAA CGG GAA G-3', reverse: 5'-GGT GAA GAC GCC AGT GGA CT-3', probe: ATC CCA TCA CCA TCT TCC AGG AGC G; human Trx-1 forward: 5'-AAG CCT TGG ACG CTG CAG-3', reverse: 5'-CAT CCT GAC AGT CAT CCA CAT CTA CT-3', probe: TGA TCA AGC CTT TCT TTC ATT CCC TCT C; human TRPC1 forward: 5'-AGC GCA TGT GGC AAT CTT TG-3', reverse: 5'-TTG CCA CCA GCA GTT TGG TA-3'; human HIF-1 α forward: 5'-ACA GCA GCC AGA CGA TCA TGC AG-3', reverse: 5'-AAC TGG TCA GCT GTG GTA ATC CAC T-3'; human COX-2 forward: 5'-TGC ATT CTT TGC CCA GCA CT-3', reverse: 5'- AAA GGC GCA GTT TAC GCT GT-3'; human TNF- α forward: 5'-GCC CAG GCA GTC AGA TCA TC-3', reverse: 5'-CGG TTC AGC CAC TGG AGC T-3'. T-3'. Reaction mixtures containing Premix Ex TaqTM (TaKaRa code: DRR039) and SYBR Green (TaKaRa code: DRR041; TaKaRa Biotechnology, China) were prepared according to the manufacturer's protocol. GAPDH was used as an internal standard for all samples.

Statistical analyses

Data was expressed as mean \pm SD values. Statistical analysis was performed by SPSS software. A one-way analysis of variance followed by a post hoc comparisons test was performed. Comparisons between PE and normal group was analyzed by multiple-samples comparison. Differences was considered statistically significant at either *P<0.05. The related variables was later compared by nonparametric Spearman's correlations and step-wise linear regressions analysis. Statistical significance is set at *P<0.05.

Results

The expression of TRPC1 in cerebral cortex and peripheral blood in mice, and blood of patients with ischemic stroke

In the acute phase of cerebral ischemia, the overload of intracellular Ca^{2+} is an important factor. TRPC1 is an ion channel located on the plasma membrane of numerous human and animal cell types. After 2 h of MCAO, we extracted the cortex and peripheral blood from the mice. We found that the mRNA level of TRPC1 was increased in mice both in cortex and peripheral blood by qRT-PCR (Fig.1A,B), as well as the mRNA level of TRPC1 was significantly increased in patients with ischemic stroke compared to the normal group (Fig. 1C). This result suggest that TRPC1 is induced by ischemia in brain.

The expression of HIF-1 α in cerebral cortex and peripheral blood in mice, and blood of patients with ischemic stroke

The inducible factor 1 α (HIF-1 α) rapidly increases under hypoxia, systemic hypoxia and cerebral ischemia. HIF-1 α is induced by cellular ischemia and hypoxia. We found that the mRNA level of HIF-1 α was increased both in cortex and peripheral blood of mice by qRT-PCR (Fig.2A, B). At the same time, we found that the mRNA level of HIF-1 α was significantly increased in patients with ischemic stroke compared to the normal group (Fig.2C)

The expression of Trx-1 in cerebral cortex and peripheral blood in mice, and blood of patients with ischemic stroke

Trx-1 is induced by ischemic stress and plays a cytoprotective role against cellular damage and oxidative stressful perturbations. We found the mRNA level of Trx-1 was significantly increased both in the brain cortex and peripheral blood in mice by qRT-PCR (Fig.3A, B). Also, we found that the mRNA level of Trx-1 was significantly increased in patients with ischemic stroke compared to the normal group (Fig.3C).

The expression of TNF- α in cerebral cortex and peripheral blood in mice, and blood of patients with ischemic stroke

Cerebral ischemia also triggers a series of the inflammatory cascade reactions. The studies have examined the peripheral blood mononuclear cells to determine gene expression in cerebral system[11]. We found that TNF- α was significantly increased both in the cortex and peripheral blood in mice (Fig.4A, B) too. Also we found that the mRNA level of TNF- α was significantly increased in patients with ischemic stroke compared to the normal group (Fig. 4C).

The expression of COX-2 in cerebral cortex and peripheral blood in mice, and blood of patients with ischemic stroke

COX-2 is up-regulated by the inflammatory response and is related with TNF- α . So, we examined the expression of COX-2 by qRT-PCR. We found that the mRNA level of COX-2 was significantly increased both in cortex and peripheral blood in mice (Fig.5A, B). Also we found that the mRNA level of COX-2 was significantly increased in patients with ischemic stroke compared to the normal group (Fig.5C).

The relationships among TRPC1 and ischemia related molecules

Since the above molecules detected in this study are found to be increased after the ischemic stroke and the changing trends of these molecules are similar, it is interesting to analyze the relationships among these molecules. We compared the relationships among these molecules expressions to identify related variables by nonparametric Spearman's correlations and step-wise linear regressions analysis. The expression of TRPC1 is related with expression of HIF-1 α , or Trx-1, or TNF- α , or COX-2 (Fig. 6A-D).

Discussion

Ischemic stroke is one of the most lethal diseases, causing disability and death in the world[12]. In the acute phase of cerebral ischemia, the overload of intracellular Ca²⁺ is an important factor that causes the death of neuron. Transient receptor potential channel 1 (TRPC1) is a nonselective cation channel located on the membrane and is highly permeable to Ca²⁺[13-15]. TRPC activation initiates Ca²⁺ entry pathways and is essential for maintaining cytosolic, endoplasmic reticulum (ER), and mitochondrial Ca²⁺ levels. Recent years great progress has been made in elucidating

1 their involvement in health and disease[15]. Up-regulation of TRPC1 appears to be a
2 general feature of smooth muscle cells in occlusive vascular disease[16] and is
3 correlated with changes in vascular smooth muscle (VSM) proliferation and
4 hypertrophy[17]. Our result showed that TRPC1 mRNA was increased in cortex,
5 peripheral blood in mice and patients with ischemic stroke (Fig. 1). However, the role
6 of TRPC1 in regulating Ca^{2+} homeostasis after ischemia stroke is needed to be further
7 studied.
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10 We further examined the molecules related with ischemia. HIF-1 α is induced
11 under reduced oxygen level in the brain after cerebral ischemia. Systemic hypoxia
12 rapidly increases the nuclear content of HIF-1 α in mice brains[18, 19]. Hypoxia
13 induces expression of HIF-1 α . Our result showed that the mRNA level of HIF-1 α was
14 increased in the cortex and peripheral blood in mice of MCAO model and patients
15 with ischemic stroke (Fig. 2).
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18 The initiating factor of angiogenesis in hypoxic environment is associated with
19 oxidative stress. ROS is the key factor that damages the neuronal lipids and proteins
20 during cerebral ischemia[20, 21]. Trx-1 is increased by ROS, such as H_2O_2 [22] and
21 reperfusion after ischemia[23]. Thus, Trx-1 possesses a neuronal protective effect
22 through anti-oxidative, and resistance to hypoxia[24]. The mRNA level of Trx-1 was
23 increased both in cortex and peripheral blood in mice after MCAO, and also Trx-1
24 was increased in peripheral blood of patients with ischemic stroke (Fig. 3).
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27 It has been reported that inflammatory reaction is induced during ischemic stroke.
28 The higher concentrations of plasma pro-inflammatory factors are associated with
29 rapid neurological deterioration and poor functional outcome in stroke patients[25,
30 26]. COX-2 is an inducible enzyme that is up-regulated by the inflammatory
31 responses[27]. TNF- α was increased in brain following ischemia onset[28], as well as
32 in peripheral blood[29]. The expressions of TNF- α and COX-2 were increased in
33 cortex, peripheral blood in mice of MCAO model and patients with ischemic stroke
34 (Fig. 2, 3). Our result is consistent with previous reports.
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37 Although several molecules expressions were examined in the present study,
38 however, whether these genes are related or not remains unclear. We analyzed the
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relationships among TRPC1 with HIF-1 α , or Trx-1, or TNF- α , or COX-2 in peripheral blood in patients. The data showed TRPC1 was closely related with expression of HIF-1 α , or Trx-1, or TNF- α , or COX-2 (Fig.6). It has been reported that TRPC1 activity was regulated by redox activity of Trx-1[30]. Trx-1 is a cofactor of nerve growth factor[31]. Since Ca²⁺ influx via TRPC channels appears to be a critical component of the signalling cascade that mediates the guidance of growth cones and survival of neurons in response to chemical cues such as neurotrophins or Netrin-1[32]. Thus, TRPC1 may be a downstream of Trx-1 after ischemic stroke. Our previous study showed Trx-1 is closely related with HIF-1 α , TNF- α , or COX-2[33], thus TRPC1 induction may be also signal pathway involved in ischemia reaction.

TRPC1 is expressed itself in the central nervous system. TRPC1 is also expressed in T cells[34]. Evidence that TRPC1 also forms a stretch-activated channel was recently presented [7]. Neuronal TRP channels has been reported as thermometers, pathfinders and life-savers[8]. However, TRPC1 activation by ischemia in brain and peripheral blood has not been reported yet. To our knowledge, this is the first report where TRPC1 was induced in MCAO model and ischemic stroke. Thus, TRPC1 may be taken as an ischemia-meter.

Conclusion: The mRNA level of TRPC1 was induced after ischemia in mice and in patients with ischemic stroke. The expression of TRPC1 is closely associated with expressions of ischemia related molecules in brain and peripheral blood of mice of MCAO model and patients with ischemic stroke. Thus, TRPC1 may be used as a ischemic sensation in mice and patients with focal cerebral ischemia.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Figure legends

Figure.1 The expression of TRPC1 in cerebral cortex and peripheral blood in mice of MCAO model and blood of patients with ischemic stroke

(A, B)The mRNA level of TRPC1 in cortex and peripheral blood in mice by qRT-PCR (n=6) (P<0.05). (C)The mRNA level of TRPC1 in patients with ischemic stroke and normal group (n=30) (P<0.05).

Figure.2 The expression of HIF-1 α in cerebral cortex and peripheral blood in mice of MCAO model and blood of patients with ischemic stroke

(A, B)The mRNA level of HIF-1 α in cortex and peripheral blood in mice by qRT-PCR (n=6) (P<0.05). (C)The mRNA level of HIF-1 α in patients with ischemic stroke and normal group (n=30) (P<0.05).per group.

Figure.3 The expression of Trx-1 in cerebral cortex and peripheral blood in mice of MCAO model and blood of patients with ischemic stroke

(A, B)The mRNA level of Trx-1 in cortex and peripheral blood in mice of MCAO

1 model by qRT-PCR (n=6) (P<0.05). (C)The mRNA level of Trx-1 in patients with
2 ischemic stroke and normal group (n=30) (P<0.05).
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4 **Figure.4 The expression of TNF- α in cerebral cortex and peripheral blood in**
5 **mice of MCAO model and blood of patients with ischemic stroke**
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7 (A, B)The mRNA level of TNF- α in cortex and peripheral blood in mice by qRT-PCR
8 (n=6) (P<0.05). (C)The mRNA level of TNF- α in patients with ischemic stroke and
9 normal group (n=30)(P<0.05).
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11 **Figure.5 The expression of COX-2 in cerebral cortex and peripheral blood in**
12 **mice of MCAO model and blood of patients with ischemic stroke.**
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14 (A, B) The mRNA level of COX-2 in cortex and peripheral blood in mice by
15 qRT-PCR (n=6) (P<0.05).(C)The mRNA level of COX-2 in patients with ischemic
16 stroke and normal group (n=30) (P<0.05).
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18 **Figure.6 The relationships among TRPC1 and HIF-1 α ,Trx-1 , TNF- α , COX-2**
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20 The relationships among these genes expressions in patients with ischemic stroke.
21 (A)TRPC1 is related with HIF-1 α (α =0.641, P<0.01). (B)Trx-1 (α =0.836, P<0.01). (C)
22 and(D) TNF- α (α =0.554, P<0.01), COX-2(α =0.640, P<0.01).
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