Cytomembrane ATP-sensitive K⁺ channels in neurovascular unit targets of ischemic stroke in the recovery period

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Abstract. The present study was to analyze the mechanism of cytomembrane ATP-sensitive K⁺ channels (KATP) in the neurovascular unit treatment of ischemic stroke in the recovery period. A total of 24 healthy adult male Wistar rats of 5-8 weeks age, weighing 160-200 g were randomly divided into the control (sham-operation group), model, KATP blocker and KATP opener groups (n=6 rats per group). Nylon cerebral artery occlusion was conducted using nylon monofilament coated with Poly-L-lysine, which was used to produce a cerebral infarction model. After feeding normally for 3 days, 5-hydroxydecanoate (40 mg/Kg), and diazoxide (40 mg/Kg) were injected to the abdominal cavity in the blocker, and opener groups, respectively. The control received an equivalent normal saline that was injected into the sham-operation and model groups. The animals were mutilated and samples were collected after 3 days. RT-PCR was used to detect the expression levels of the three subunits of KATP, i.e., Kir6.1, and sulfonylurea receptor (SUR) 1 and SUR2 mRNA, as well as to calculate infarct size in tetrazolium chloride staining. The expression level of mRNA in the opener group were significantly higher, followed by the model and blocker groups, with the control group being the lowest (P<0.05). Infarct size in the opener group was markedly smaller than the model and blocker groups, and infarct size in the blocker group was significantly larger (P<0.05). Thus, the target treatment on KATP may improve the prognosis of ischemic stroke during the recovery period.

Introduction

The structural foundation of the neurovascular unit (NVU) includes neuron, blood brain barrier (BBB), including endothelial cells, basement membrane, foot process of astrocyte and pericyte), microglia and extracellular matrix, which maintain completeness of brain tissues, of which BBB is a core component of NVU (1).

Patients with cerebral arterial thrombosis experience vasculopathy initially, followed by nervous system lesions with corresponding symptoms. Protective treatment for neurons only may have limited effects, resulting in possibility of treatment being lost (2). NVU constitutes a hot spot and has become the focus of current studies on thrombosis. NVU involves multiple structures, multiple action segments and complex networks of many signal channels, and an increasing number of studies have discussed it extensively from different aspects. Among these studies, complication occurrence, interaction and final targets of treatment strategies are to interfere with signal channels (3), thus studies on signal channels may have broad and attractive prospects. ATP-sensitive potassium channels (KATP) are widely distributed in the central nervous system and belong to ligand-gated voltage-independent inwardly rectifying K⁺ channels. The main function of KATP is to couple cellular energy metabolism and electrophysiological activities, and it plays important roles in the occurrence of thrombosis, ischemic preconditioning and reperfusion injury (4). At present, studies have focused on the regulatory roles of KATP channels in myocardial ischemic injury, movement of smooth muscle and skeletal muscle and pancreatic secretion (5,6). However, to the best of our knowledge, there are few studies on thrombosis. An important mechanism of pathological changes in thrombosis is energy metabolism dysfunction (7). Consequently, it has been hypothesized that KATP channels play an important role in energy metabolism of thrombosis.

The aim of the present study was to analyze the mechanism of cytomembrane ATP-sensitive K⁺ channels (KATP) in the neurovascular unit treatment of ischemic stroke during the recovery period.

Materials and methods

Animals. Fifteen healthy adult Wistar male rats, aged 5-8 weeks and weighing 160-200 g, were provided by the Shanghai Laboratory Animal Research Center (Shanghai, China). The animals were placed in an environment of room temperature.
22±0.5°C, with 12 h of light/dark cycle with unlimited access to food and water.

The main reagents used were 5-hydroxydecanoate (5-HD) and diazoxide, which were purchased from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). A TRIzol extraction kit was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), and an RT-PCR kit from MBI Fermentas, Inc. (Burlington, ON, Canada). The DNA Marker DL2000 was obtained from Shenzhen JINGMEI Engineering Biology Co., Ltd. (Shenzhen, China), and tetrazolium chloride (TTC) from Henan Huamei Biological Engineering Co., Ltd. (Henan, China).

The main instruments used were tissue homogenizer and PCR amplification instrument 2400 (both from Ningbo Technology Co., Ltd., Ningbo, China), ultraviolet spectrophotometer and high-speed tabletop refrigerated centrifuge manufactured by Beckman Coulter, Inc. (Brea, CA, USA), electronic analytical balance manufactured by OHAU Company (Parsippany, NJ, USA), VDS ultraviolet gel imaging analysis system was purchased from Pharmacia AB (Stockholm, Sweden), THZ-C constant temperature oscillator from Jiangsu Taijiang Experimental Equipment Factory (Jiangsu, China), and ultra-low temperature freezer from Sanyo Electric Co., Ltd. (Tokyo, Japan).

**Preparation of middle cerebral artery occlusion (MCAO) model.** Rats were deprived of food and water for 12 and 4 h, respectively, chloral hydrate was used to anesthetize the abdominal cavity. The operating area was sterilized, and an incision of approximately 8 cm was made in the middle section of neck. The skin and muscle were bluntly dissected to expose left carotid artery, and the internal and external carotid were separated along the anatomic structure. The nylon mono-filament coated with Poly-L-lysine was used to insert into the internal carotid via the incision, and the direction of nylon thread was adjusted continuously. When approaching bifurcation of internal and external carotid, forward resistance was felt which meant that the origin of middle cerebral artery occlusion was achieved, the nylon thread was placed for 4 h and muscle and skin were sutured by layers.

The experiment was completed after 4 h, the nylon thread was removed and the whole layer was sutured (Fig. 1). Headlamps were used to light rats in the experiment and to ensure that their body temperature was at 37°C and penicillin was used to prevent infection. When rats became awake after anaesthesia, it was observed that side limbs could not be abducted completely, muscular tension decreased and the animals fell to the contralateral side spontaneously. The contralateral limbs. In addition, movement decreased and the rats circled to the contralateral side. After anaesthesia, it was observed that side limbs could not move completely, muscular tension decreased and the animals fell to the contralateral side when standing, i.e., fore limbs. In addition, movement decreased and the rats circled to the contralateral side spontaneously. The contralateral limbs were incapable of collapsing when the tails were lifted, and resistance weakened, indicating the model was successfully created.

**Experimental groups.** The animals were randomly divided into the control (sham-operation group), MCAO, K$_{ATP}$ blocker and K$_{ATP}$ opener groups. Each group included 6 rats. At 3 days after successfully establishing the models, 5-HD (40 mg/Kg) was injected into the abdominal cavity in the K$_{ATP}$ blocker group, and diazoxide (40 mg/Kg) was injected into the abdominal cavity of the opener group. Surgery was performed to expose the carotid artery and suture by layers in the sham-operation group, and the equivalent volume of normal saline was injected in the sham-operaton and model groups. The animals were mutilated and samples were collected after feeding normally for 3 days.

**Observation indices and detection methods.** The expression level and infarct size of three subunits of K$_{ATP}$, i.e., Kir6.1, sulfonylurea receptor (SUR) 1 and SUR2 mRNA were compared in the different groups.

**RT-PCR for detection of expression of subunits.** A TRIzol extraction kit was used to extract total RNA of brain tissues according to the manufacturer’s instructions. RNA was assessed for purity and concentration using a UV-spectrophotometer. The PCR steps consisted of primer designing by DNAstar 7.0, which were produced by Invitrogen Life Technologies. For reverse transcription (RT), 4 µg of total RNA was added to 4 µl 5X RT buffer solution, 2 µl dNTP, 1 µl RNAase inhibitor, 1 µl random primer, 1 µl M-MLV RT, DEPC-treated water to produce the final volume as 20 µl. PCR reaction conditions were for 10 min at 25°C, 60 min at 42°C, 10 min at 70°C, and place RT product at -20°C to preserve. For cDNA amplification, 4 µl reverse-transcribed product was added to 12.5 µl 2X PCR buffer solution and 1 µl specific primers, to produce the final volume of 25 µl with H$_2$O.

Reaction conditions of β-actin were: denaturation at 94°C for 5 min, annealing at 94°C for 45 sec, at 60°C for 45 sec,
at 72˚C for 45 sec, and extension at 72˚C for 5 min, which accounted for 35 cycles. Reaction conditions of Sur1 and Sur2 were: denaturation at 95˚C for 15 min, denaturation at 95˚C for 30 sec, annealing 57˚C for 45 sec, extension at 72˚C for 45 sec, and a final extension at 72˚C for 5 min, which accounted for 37 cycles. For electrophoresis, PCR product was loaded on 1.5% agarose gel electrophoresis (95 mA, 30 min) and images were captured with a gel imaging system. Using a scanning densitometer (KS400v 3.0) (Beijing Maisiqi High-tech Co., Ltd., Beijing, China; http://www.msdyq.cn/), the ratio of electrophoresis strips and expression of gray level of internal control β-actin strip were estimated. Detailed primers for RT-PCR are shown in Table I.

Calculation of infarct size in TTC staining. After general anesthesia, the rats were decapitated and brains were removed. The olfactory bulb and lower brain stem were separated, and carefully removed. The brain was sectioned into five 2 mm pieces after deep freezing for 20 min at -20˚C. The tissues were added to TTC buffer solution, stained for 30 min using 4% paraformaldehyde buffer solution to fix and images were captured with high-resolution cameras. Normal brain tissue was red or pink while the infarct areas were white. Using Photoshop image processing software, TTC staining images were obtained and analyzed. The corresponding measuring instruments were used to calculate the infarct size, and the results were shown as area percentage of the homolateral brain tissues.

Statistical analysis. SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used to statistically analyze the data. Data were presented as mean ± standard deviation. The single-factor analysis of variance was used for comparison of groups, and α<0.05 was taken as the inspection level. LSD and Bonferroni tests were used for comparisons between two groups, by taking 1/4α<0.0125 as inspection level.

Results

mRNA expression level of three subunits of K<sub>ATP</sub>. Among the expression levels of the three subunits of K<sub>ATP</sub>, the expression level of mRNA in the opener group was significantly higher, followed by the model and blocker groups, with the control group being the lowest (P<0.05; Figs. 2 and 3).

| Gene      | Sequence no. | Primer sequences                      | Size  | Annealing  | Cycles |
|-----------|--------------|---------------------------------------|-------|------------|--------|
| Kir6.1    | D88159       | 5'-ACCAGAATTCTCTGCCGGAAG-3' 5'-GCCCTGAACTGGTGATGAT-3' | 297 bp| 60˚C 45 sec | 35     |
|           |              | 5'-GGAGCACAATCCAGACCAAGAT-3' 5'-AGGAGCAGAATGATGACAG-3' | 249 bp| 57˚C 45 sec | 37     |
| SUR1      | L40624       | 5'-CCATCATCAGTGTCAAAAAGC-3' 5'-GGCTGCTTCTCCTGTATTGGTA-3' | 148 bp| 57˚C 45 sec | 37     |
| SUR2      | AC108508     | 5'-AAGTACCCCCATGTAAACCGG-3' 5'-ATCACAAATGCCGTTAC-3' | 257 bp| 60˚C 45 sec | 22     |
| β-actin   | NM031144     | 5'-AAGTACCCCCATGTAAACCGG-3' 5'-ATCACAAATGCCGTTAC-3' | 257 bp| 60˚C 45 sec | 22     |

SUR, sulfonylurea receptor.

Figure 2. Expression level of K<sub>ATP</sub>’s three subunits, kir6.1, SUR1 and SUR2 mRNA. SUR, sulfonylurea receptor.

Figure 3. Relative mRNA expression levels for K<sub>ATP</sub>’s three subunits among different groups. SUR, sulfonylurea receptor.
Table II. Infarct size (%).

| Group    | Infarct size |
|----------|--------------|
| Opener   | 20.3±8.4     |
| Model    | 46.2±13.5    |
| Blocker  | 75.8±19.4    |
| F        | 7.825        |
| P-value  | <0.001       |

Infarct size in the opener group was smaller than that in the model and blocker groups, and infarct size in the blocker group was the largest. The difference was statistically significant (P<0.05; Fig. 4 and Table II).

Discussion

The recovery period of cerebral arterial thrombosis often refers to 15 days to half year of morbidity, and previous findings have shown that this period is crucial for the restoration of nerve function of thrombosis (8), which is directly associated with long-term prognosis of thrombosis. Many theories including synaptic plasticity, axonal sprouting, denervated supersensitivity, nervous centralis bilateral innervation on movement, study, memory, regional functional reorganization and abundant environmental stimulus, emphasize the important role of NYU in the recovery period of thrombosis (9).

The K\textsubscript{ATP} channels, including those from cell membrane and mitochondrial membrane, are heteromultimers comprising two types of subunits: Four inwardly rectifying K\textsuperscript{+} channel subunits (Kir6.1 and Kir6.2) and four SUR subunits, which are members of the family of ATP-binding cassette transporter proteins. The former forms ion channels and the latter determines K\textsubscript{ATP} function (10) (Fig. 5).

At present, SUR is considered to be the main target of drug action, which can increase the sensitivity of neuron Kir6.1 to sulfonylurea drugs and channel opener, as well as to ATP (11). Griesemer \textit{et al} showed that there are many K\textsubscript{ATP} channels in pyramidal cells and intermediate neurons of different regions of the hippocampus (12).

Qu \textit{et al} applied diazoxide (K\textsuperscript{+} channel opener) to the MCAO rat model as a pretreatment and found that, the cortical infarct area may be significantly reduced by 65%, and that neuronal apoptosis decreased while astrocyte activation increased (13). The results of the present study showed that the mRNA expression level of the three K\textsubscript{ATP} subunits in the opener group was the highest, followed by the model and blocker groups. The results were statistically significant as compared to the control group, which had the lowest expression level.

Infarct size in the channel opener group was significantly smaller than that in the model and blocker groups, and was statistically significant, while the blocker group had the largest infarct size. The suture method contributed to less model injury and a higher survival rate. Under a light microscope the pathological changes of infarction cells are similar to those of the human body, with good model stability (14).

Current studies mainly focus on the changes of tissues and cells during acute cerebral infarction. In the present study, the intervention research was carried out in 3 days after modeling during rat convalescence, and the \textit{in vitro} study was conducted 3 days after drug intervention, which basically simulated the function of NVU target in cerebral infarction convalescence (15).

In addition, the results have shown that many K\textsuperscript{+} channels were active during cerebral infarction convalescence and in the blocker group markedly reduced its activity compared to the opener group, thereby increasing its expression, and influencing the area of infarction, which closely connected with the K\textsuperscript{+} channel opening degree.

The activity of K\textsubscript{ATP} channel is affected by the concentration of intracellular ATP/ADP (16). Previous findings have shown that Kir subunit had binding sites of ATP (17), with the most effective endogenous blocker being achieved through ATP ligand activity but not Mg\textsuperscript{2+}. ADP and other nucleoside...
diphosphates serve as endogenous agonists to K\textsuperscript{+} channel (18). Therefore, the proportion of ATP and ADP is crucial to the regulation of K\textsubscript{ATP} channel activity, and also is an essential regulatory factor to link channel activity with cell metabolism (19). Therefore, the K\textsubscript{ATP} channel may prove an important target to improve the long-term prognosis of stroke.

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