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Original Article

Effect of activation of the Ca^{2+}-permeable acid-sensing ion channel 1a on focal cerebral ischemia in diabetic rats

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Abstract: We investigated the role of acid-sensing ion channel la (ASIC1a) expression and changes in intracellular Ca^{2+} concentration ([Ca^{2+}]) in focal cerebral ischemia after middle cerebral artery occlusion (MCAO) in a rat model of diabetes mellitus (DM). Male Wistar rats (n = 108) were divided into three groups: the MCAO, DM + MCAO, and DM + MCAO + fasudil groups (n = 36 each). Samples were obtained 1, 3, 6, and 24 h after ischemia induction (n = 9). Rats in the DM + MCAO + fasudil group were treated with 1 mg/kg fasudil, a Rho-kinase inhibitor, by caudal vein injection 30 min after MCAO was performed. ASIC1a expression gradually increased with time in the MCAO and DM + MCAO groups (0.71 ± 0.10 nM, 0.80 ± 0.11 nM, 0.86 ± 0.08 nM, 0.93 ± 0.09 nM; 0.86 ± 0.11 nM, 1.05 ± 0.51 nM, 2.42 ± 0.08 nM, 2.78 ± 0.04 nM; pairwise comparisons at each time point, $P < 0.05$), and was higher in the DM + MCAO than the MCAO group ($P < 0.05$). [Ca^{2+}] gradually increased in the DM + MCAO group (106.32 ± 18.6 nM, 137.84 ± 14.32 nM, 151.94 ± 18.38 nM, 183.61 ± 7.96 nM, $P < 0.05$). ASIC1a expression and calcium currents were reduced in the DM + MCAO + fasudil group. The overload of intracellular [Ca^{2+}] caused by ASIC1a activation could be one mechanism for the aggravation of focal cerebral ischemia in diabetes.

Keywords: Cerebral ischemia, calcium channel, brain injury

Introduction

The acid-sensing ion channel 1a (ASIC1a) plays an important role in brain ischemia [1, 2]. Application of ASIC1a blockers or genomic knockout of ASIC1a can reduce the volume of focal cerebral infarction, thus exerting a significant neuroprotective effect [3]. The role of ASICs in cerebral ischemia has attracted widespread attention (Petroff et al., 2008). The role of ASIC1a function in diabetic patients with cerebral ischemia is unclear. We explored the possible mechanisms of increased infarction volume in diabetes mellitus (DM) after ischemia induced by middle cerebral artery occlusion (MCAO) from the perspective of ASICs and intracellular Ca^{2+}, which are important for better understanding of the pathogenesis of diabetic patients with cerebral infarction and development of effective therapeutic targets.

Materials and methods

Experimental animals

Male Wistar rats (n = 108), aged 6 weeks and weighing 190-240 g, were provided by the Experimental Animal Center of Jilin University, license number SCXK (Ji) 2007-0003. Rats were housed at 20 ± 2°C with a relative humidity of 40-60% and natural light. Experiments were approved by the Animal Ethics Committee of Jilin University.

Grouping

Rats were divided into three groups: a MCAO group, DM + MCAO group, and DM + MCAO + fasudil group (n = 36 each). Blood samples were obtained at 1, 3, 6 and 24 h after ischemia (n = 9).

Diabetic animal model

In accordance with a previous study [4], rats were kept on a high-fat diet for 4 weeks, and then streptozotocin (STZ, Sigma, USA) solution (40 mg/kg in citrate buffer prepared at pH 4.2) was injected via tail vein. Rats were fed for 7 d, and then 1 d before MCAO operation, rats were deprived of food and water overnight. Blood was collected from the tail vein the following morning, and fasting blood glucose ≥ 16.7 mM was considered successful ischemic modeling (glucose meters, blood glucose test strips, Bayer Corporation, German). Rats with glucose levels > 30 mM were excluded and their respective groups were supplemented.

Establishment of MCAO and DM + MCAO models

The Longa method was used to establish the MCAO model, and the reported 5-point method was used for evaluation [5]. A 1-3 point evaluation was the inclusion criterion; rats who had 0 or 4 points, or who did not survive, were removed from the study. After successful DM modeling for 0.5 h, rats in the fasudil group were administered with 1 mg/kg fasudil (fasudil hydrochloride, Tianjin Red Sun Company) by caudal vein injection.

Demonstration of focal cerebral infarction by hematoxylin and eosin (HE) staining

After modeling, one rat in each group at each time point was selected for HE staining. Rats were anesthetized, and the brain was removed by 0.9% saline lavaging from the left atrial appendage. After paraffin embedding, 4-μm sections were made on a brain tissue slicer (Shanghai Precision Instrument Co., Ltd.), and the degree of brain tissue damage in each group was quantified by HE staining.

ASIC1a protein expression quantification by Western blot

Ischemic cortical tissue from the left hemisphere of MCAO, DM + MCAO, and DM + MCAO + fasudil rats were obtained at different time points and cut into small pieces. Then, 150-250 μl lysis buffer was added per 20 mg of brain tissue. After homogenization in a high speed homogenizer, lysis, and centrifugation at 1204 × g for 3-5 min, the supernatant was collected and mixed with 5 × SDS sample buffer at a ratio of 4:1, and subjected to shaking and boiling for 3-5 min. Then the solution was cooled to room temperature and centrifuged (1003 × g) for 30 s and separated by electrophoresis. After blocking in 20 ml blocking buffer for 3 h, an anti-ASIC1a antibody (1:500, Santa Cruz Biotechnology, Inc., US) was added at 4°C overnight. After TBS wash, goat anti-rabbit antibody (Wuhan Boster) was diluted to 1:500 and labeled with horseradish peroxidase, and then oscillated with the cellulose membrane at room temperature for 3 h in a hybridization bag; The membrane was washed by TBS; DAB (Santa Cruz Biotechnology, Inc., US) was used for staining. Gray bands were measured with Bandscan (US Biotech Inc.), with the ratio of ASIC1a to β-actin intensities in each column as the expression level of ASIC1a protein in each group at different time points.

[Ca^{2+}] detection in brain ischemia tissues

Rats in the MCAO, DM + MCAO, and DM + MCAO + fasudil groups were decapitated and the brains were collected at the corresponding ischemic time points. They were then quickly moved into the incubation medium (0-4°C) for 30-60 s. Once on the ice tray, 2/3 of the ischemic cortical tissue in the middle of the left cerebral hemisphere was extracted and cut to 400-μm sections using a vibratome. The sections were placed in the incubation medium and were incubated for 50-60 min at room temperature while the solution was bubbled with 95% O_2/5% CO_2, and were then transferred to a Pronase digestive solution at 0.4 mg/ml at 32°C for 30 min. Gentle pipetting was conducted to obtain a single cell suspension, which was filtered through a mesh filter and incubated at 37°C for 30 min, washed with Hank's Balanced Salt Solution (Beijing Huamai New Technology Co., Beijing, China) twice, and then centrifuged. The supernatant was discarded, and the precipitate was resuspended in 0.5% BSA-DMEM; Fura-2/AM (Shanghai Microcrystalline Co.) was added and the suspension was adjusted to a final concentration of 10 μM. The solution was continuously bubbled with mixed gas throughout the process. Centrifugation was performed again, and cells were resuspended and adjusted to a cell density of 5 × 10^5 cells/mL with HEPES buffer. According to the formula: the intracellular calcium concentration $[Ca^{2+}]_i = K_d (F_0-F_{min})/(F_{max}-F_0)$; where F_0 is the measured fluorescence value; F_{min} is the

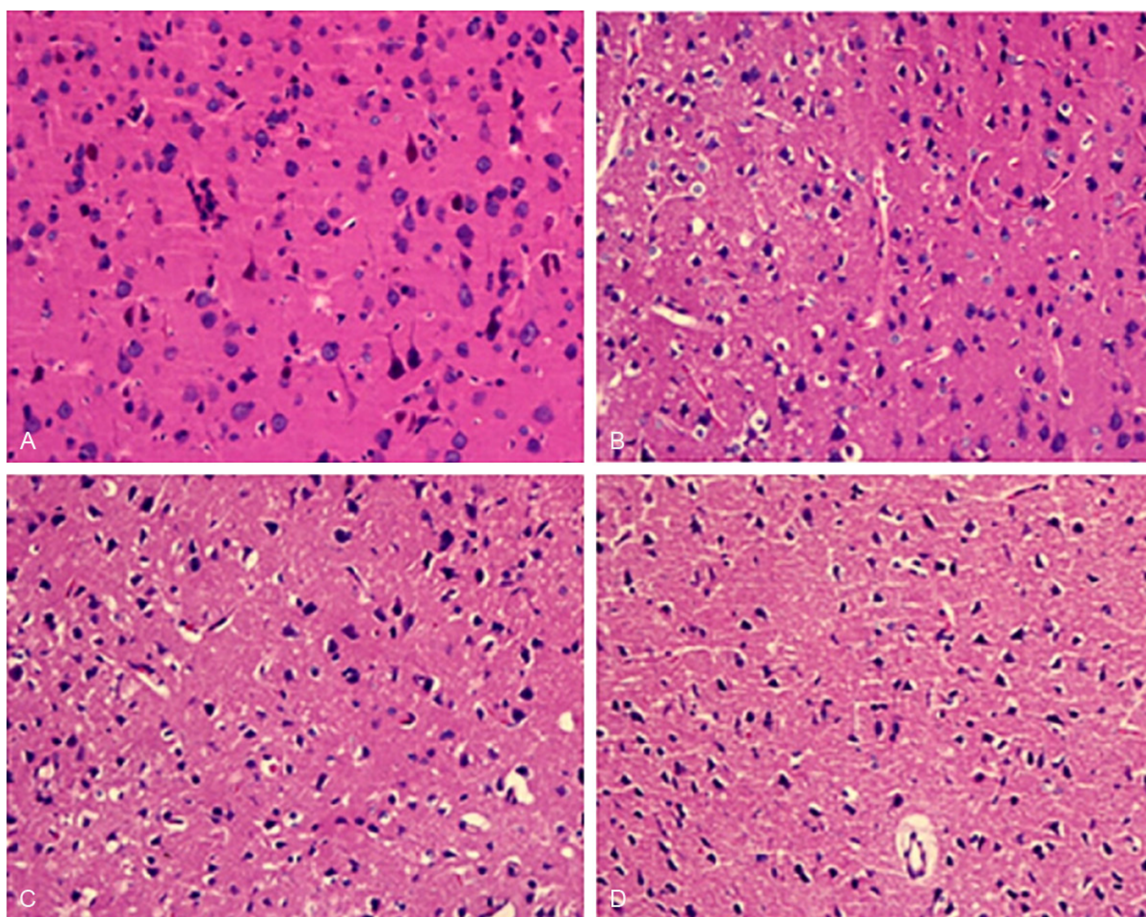

Figure 1. The degree of brain damage in each group shown with HE staining (200 ×).

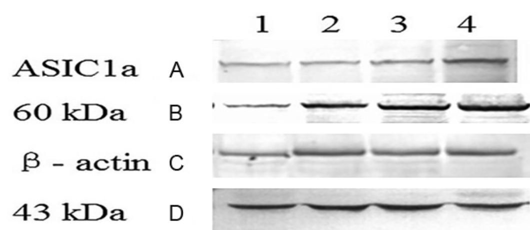

Figure 2. ASIC1a protein from each group at four different ischemic times. Note: row A: MCAO group, row B: DM + MCAO group, row C: DM + MCAO + fasudil, row D: Indicated Beta-Actin; Columns 1-4: 1, 3, 6, and 24 h, respectively, after ischemia induction.

minimum fluorescence after the addition of EGTA; F_{max} is the maximum fluorescence intensity by adding 0.09% Triton X-100 at 1 M $CaCl_2$; and K_d is a constant (224 nM). One milliliter cell suspension was added to a cuvette, and a fluorescence spectrophotometer (F-7000, Hitachi) was used to measure $[Ca^{2+}]_i$. The fluorescence intensity of each group was measured with a 500-nm emission wavelength and a 300-380-nm excitation wavelength. Fluorescence intensity peaked at about 330-340 nm, indicating that Fura-2/AM had been loaded into the cell. An excitation wavelength was used to detect intracellular Ca^{2+}, which was substituted into the above formula to calculate $[Ca^{2+}]_i$.

Statistical methods

SSPS 14.0 was used for all statistical analyses. Samples were compared by the Kolmogorov-Smirnov test and homogeneity of variance analysis. Differences between the two groups were compared by the Student's *t*-test; data in multiple sets were compared using analysis of variance (ANOVA). Data are presented as mean ± SD.

Results

Neurological scores of each group

Fasting glucose levels of all diabetic rats the morning after MCAO was 21.7 ± 2.3 mM, which

Table 1. $[Ca^{2+}]_i$ at different ischemic time points (mean ± SD, nM)

Group	# of cases	1 h	3 h	6 h	24 h
DM + MCAO	4	106.32 ± 18.63	137.84 ± 14.32	151.94 ± 18.38	183.61 ± 7.96
DM + MCAO + fasudil	4	87.94 ± 6.28	103.34 ± 11.17	128.67 ± 6.72	148.57 ± 12.43
t value		1.87	3.78	2.37	4.75
p value		0.111	0.009	0.049	0.003

Note: DM + MCAO: diabetic cerebral ischemia group; DM + MCAO + fasudil: diabetic cerebral ischemia + fasudil intervention group.

is greater than the 16.7 mM diagnostic criterion for diabetes (Chen et al., 2005).

Neurological evaluation of rats at 24 h showed that DM + MCAO rats awoke much later after the operation, and the limb paralysis degree was more severe than that of the MCAO group. The extent of limb paralysis in DM + MCAO + fasudil was far less than the other two groups ($P < 0.001$). Neurological scores of focal cerebral ischemia at 24 h in the MCAO, DM + MCAO, and DM + MCAO + fasudil groups were 1.56 ± 0.73, 2.67 ± 0.50, and 1.33 ± 0.50 points, respectively ($P < 0.001$).

The extent of ischemic brain damage in each group at 24 h by HE staining

In normal brain tissues, the number and morphology of nerve cells were normal, and there was no ischemia or necrosis (**Figure 1A**). In the MCAO group, staining 24 h after ischemia showed light HE staining in the ischemic core. In these animals, brain tissue was sparser, nerve cells were fewer and smaller, condensation of the nucleus and loss of structural integrity was observed, and vacuolation was observed around cells (**Figure 1B**). In the DM + MCAO group, staining 24 h after ischemia showed significant cell death loss of normal structure, and the degree of injury was heavier than that in the MCAO group (**Figure 1C**). In the DM + MCAO + fasudil group, staining 24 h after ischemia showed that, while nerve cells were smaller and tissue was loose and showed vacuolar changes, the extent of neuronal cell damage was lighter than that in the DM + MCAO group (**Figure 1D**).

Expression changes of ASIC1a detected by Western blot at different ischemic time points

In **Figure 2**, row D contains β-actin bands, while the upper bands are from the experimental groups (see legend). At the ischemic time points of 1, 3, 6, and 24 h (corresponding to columns 1-4), the corresponding gray values of MCAO were respectively 0.71 ± 0.10, 0.80 ± 0.11, 0.86 ± 0.08, and 0.93 ± 0.09. The same values for the DM + MCAO group were 0.86 ± 0.11, 1.05 ± 0.51, 2.42 ± 0.08, and 2.78 ± 0.04. Finally, for the DM + MCAO + fasudil group, the values were 0.64 ± 0.06, 0.89 ± 0.09, 0.98 ± 0.10, and 1.03 ± 1.12. With the prolonged ischemic time, ASIC1a expression in the MCAO and DM + MCAO groups gradually increased ($P < 0.05$), and expression in the DM + MCAO group was higher than that of MCAO group ($P < 0.05$). After application of the calcium antagonist fasudil, ASIC1a expression levels in the DM + MCAO + fasudil group were reduced by varying degrees, and were significantly lower than the other groups ($P < 0.05$, shown in **Figure 2**).

Changes in $[Ca^{2+}]_i$ at different ischemic time points

Measurements of $[Ca^{2+}]_i$ surrounding ischemic cortex at 1, 3, 6, and 24 h in the DM + MCAO and DM + MCAO + fasudil groups showed that with prolonged ischemic time, $[Ca^{2+}]_i$ in the DM + MCAO group was increased (between the two groups, $P < 0.05$). In the presence of fasudil, $[Ca^{2+}]_i$ at each time point was reduced compared with that in the DM + MCAO group (**Table 1**).

Discussion

In acute cerebral ischemia, the increased hydrogen ion concentration due to acidosis activates ASIC1a channels and aggravates brain damage. The symptoms of diabetes combined with focal cerebral ischemic are severe, and the molecular mechanisms are unclear. It is not clear whether there is increased expression of ASIC1a in this particular metabolic abnormality of diabetes. In this study, a high-fat diet combined with the STZ diabetic animal model was used to study the relationship

between diabetes and ischemia. The induction of cerebral ischemia was performed by the MCAO suture method. By neurological scoring and HE staining, we confirmed that focal cerebral ischemia in diabetic rats resulted in large lesions, and that these injuries were serious. The results of western blotting experiments showed that with prolonged ischemia, the expression levels of ASIC1a in the MCAO and DM + MCAO groups were increased. This indicates that increases in ASIC1a due to acidosis occur in ischemia independent of comorbid diabetes. With prolonged ischemia, the opening of ASIC1a increases, which is consistent with Waring's results [6]. Compared with the MCAO group at each time point, the expression of ASIC1a was significantly increased in the DM + MCAO group, indicating that ASIC1a opens more under high glucose conditions. Studies have shown that ischemia and acidification have synergistic effects on the activation of ASIC1a [3]. Therefore, this result indirectly showed that the reason for the aggravation of cerebral ischemia in diabetic rats was that activation of the cell membrane may be associated with the increasing open of ASIC1a channels.

In order to further confirm the relationship between the activation of ASIC1a and the aggravation of cerebral ischemia in diabetic rats, we examined the changes in $[Ca^{2+}]_i$ in each group at different times after ischemia induction. With prolonged ischemia in the MCAO and DM + MCAO groups, the $[Ca^{2+}]_i$ gradually increased, indicating that there were synchronous changes in ASIC1a and $[Ca^{2+}]_i$ during cerebral ischemia. The permeability ASIC1a to Ca^{2+} is due to the activation of calmodulin for CaMKII phosphorylation during cerebral ischemia and hypoxia, which then acts on the Ser478 and Ser4792 residues of the ASICla channel. In addition to an enhancement in the total current, Ca^{2+} influx increases [7]. Sources of intracellular Ca^{2+} are varied. The early transient calcium current increase is due to voltage-gated calcium channel activation. Increased ASIC1a open time promotes cell depolarization, and thus increases voltage-gated calcium currents. The intercellular transmission of information causes subsequent calcium influx, which is the major source of calcium influx excluding NMDA receptors [8]. In addition, acidification, hypoxia, and high glucose levels can also increase calcium influx. Intracellular Ca^{2+} overload can activate a series of cell toxicity events by a variety of pathological processes, leading to activation of intracellular calcium enzymes, which further activate Ca^{2+}-related signal transduction mechanisms [9], causing decomposition of three major nutrients (Glucose, protein, and vitamin), increasing anaerobic metabolism and acid production, and exacerbating tissue injury [10]. $[Ca^{2+}]_i$ in cells of the DM + MCAO group was higher than in cells of the MCAO group, indicating that overload of intracellular calcium is aggravated in diabetic cerebral ischemia. One reason could be that ASIC1a increases its opening in high glucose and acidified environments. With prolonged ischemia, the synchronized increase in ASIC1a expression and $[Ca^{2+}]_i$ aggravates cellular damage.

As a tissue acidification receptor and proton-gated cation channel, pathological roles of ASIC1a in cerebral ischemia are attracting more and more attention. Researchers often use ASIC1a-specific antibodies, the ASIC antagonist amiloride, or the ASIC desensitizing toxin PcTx1 to target ASIC1a itself as a therapy [11-12]. However, due to exclusion from penetrating the blood-brain barrier, amiloride and PcTx1 are confined to animal research. Hydroxyfasudil is a Rho-kinase inhibitor [13] and an intracellular calcium antagonist, which can block the binding of Ca^{2+} to the Ca^{2+}-binding protein calmodulin to exert a nerve protective effect [14-16]. In this study, DM rats in which an caudal vein injection of fasudil was administered 30 min after ischemia showed reduced $[Ca^{2+}]_i$ at all time points tested, indicating that fasudil can effectively prevent elevations in $[Ca^{2+}]_i$. Furthermore, the neurological score of the DM + MCAO + fasudil group was significantly better than that of the DM + MCAO group, which confirms the protective effects of fasudil in cerebral ischemia. In addition to the reduced $[Ca^{2+}]_i$ in the DM + MCAO + fasudil group at all time points, the expression of ASIC1a was also reduced, which indirectly shows that there may be more complex feedback regulation between ASIC1a and Ca^{2+}, and the specific mechanisms of this regulation need to be further explored.

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Disclosure of conflict of interest

None.

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