Effects of ATP sensitive potassium channel opener on the mRNA and protein expressions of caspase-12 after cerebral ischemia-reperfusion in rats

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Abstract: Objective To investigate effects of K_{ATP} opener on the expressions of caspase-12 mRNA and protein, and to explore the role of endoplasmic reticulum (ER) stress pathway in the mechanism of K_{ATP} opener protecting against neuronal apoptosis after cerebral ischemia-reperfusion. **Methods** Two hundred rats were randomly divided into four groups: sham operation group, ischemia-reperfusion group, K_{ATP} opener group, and K_{ATP} blocker group. The middle cerebral artery occlusion (MCAO) model was established by intraluminal suture occlusion method; neuronal apoptosis was detected by TUNEL staining. The mRNA and protein expressions of caspase-12 were detected by semi-quantitative RT-PCR and immunohistochemical staining, respectively. **Results** In ischemia-reperfusion group, K_{ATP} opener group and K_{ATP} blocker group, the number of apoptotic cells and the mRNA and protein expressions of caspase-12 gradually increased following cerebral reperfusion, and reached the peak at 24 h. In K_{ATP} opener group, The number of apoptotic cells was significantly less than that in ischemia-reperfusion group and K_{ATP} blocker group at 12 h, 24 h, 48 h and 72 h ($P < 0.05$ or $P < 0.01$); while the mRNA and protein levels of caspase-12 were significantly less than those in ischemia-reperfusion group and K_{ATP} blocker group at all times ($P < 0.05$ or $P < 0.01$). There were no differences between the ischemia-reperfusion group and K_{ATP} blocker group at each time ($P > 0.05$). **Conclusion** K_{ATP} opener may protect neurons from apoptosis following the cerebral ischemia-reperfusion by inhibiting ER stress pathway.

Keywords: ATP sensitive potassium channel; cerebral ischemia; apoptosis; endoplasmic reticulum; caspase-12

1 Introduction

Researches have shown that ATP sensitive potassium channel (K_{ATP}) openers have neuroprotective effects against cerebral reperfusion injury^[1,2]. Our previous researches demonstrated that systemic administration of pinacidil may significantly decrease neuronal apoptosis following the cerebral ischemia-reperfusion by inhibiting both death receptor and mitochondria signal pathways^[3,4]. Endoplasmic reticulum (ER) stress-induced apoptosis has recently been identi-

fied as an important signal pathway different from death receptor and mitochondria signal pathways. ER is an organelle that plays an important role in folding and processing of membrane proteins, and maintenance of intracellular calcium homeostasis^[5]. Recent evidences indicate that ER stress also plays an important role in the process of cell apoptosis. Caspase-12 is an ER-specific caspase. Disturbance of calcium homeostasis and accumulation of unfolded proteins in ER may cause ER stress, which will activate caspase-12 and subsequent caspases^[6]. Therefore ER stress pathway is characterized by caspase-12 activation. In the present study, we examined the effects of pinacidil, a K_{ATP} opener, on neuronal apoptosis and the mRNA and protein expressions of caspase-12 in rats after cerebral ischemia-reperfusion, in order to explore the signaling transduction pathway of K_{ATP} opener.

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2 Materials and methods

2.1 Experimental animals and groups Two hundred male Wistar rats weighing 250-300 g (provided by the laboratory animal center of China Medical University) were randomly assigned into four groups: sham operation group, ischemia-reperfusion group, K_{ATP} opener treatment group (K_{ATP} opener group), and K_{ATP} opener plus blocker treatment group (K_{ATP} blocker group). In each group, the rats were separated into 5 subgroups according to the different time points after ischemia-reperfusion: 6 h, 12 h, 24 h, 48 h, and 72 h ($n = 10$ in each subgroup). In each subgroup, five rats were used for immunohistochemical staining, and the other five rats were used for RT-PCR.

2.2 Animal models and drug administration The middle cerebral artery occlusion (MCAO) model was performed as previously described by Nagasawa^[7]. In brief, the right common carotid artery (CCA) was exposed through a midline incision on the neck and dissected free of surrounding nerves. A 5-cm length of nylon filament (Diameter 0.26 mm) was inserted into the common carotid artery through a small incision proximal to the bifurcation and advanced in the internal carotid artery to about 18 mm distal to the carotid bifurcation until mild resistance was indicated. Reperfusion was accomplished by withdrawing the filament at 2 h after the MCAO. The operation procedure of sham operation group was the same as the other three groups except only that the nylon filament was not ligated. Body temperature of rats was controlled at 37.0 °C with a heating lamp and rectal probe. In K_{ATP} opener group, 10 μ L of pinacidil (10 nmol / 5 μ L) was administered intracerebroventricularly 30 min before induction of cerebral ischemia. In K_{ATP} blocker group, 10 μ L of the K_{ATP} blocker glibenclamide (1 μ mol / 5 μ L) was injected intracerebroventricularly 15 min prior to administration of pinacidil, the rats in sham operation group and ischemia-reperfusion group were only injected intracerebroventricularly with the same volumes of 0.9% NaCl. At each time point, the rats used for TUNEL and immunohistochemical staining were anesthetized with 10% chloral hydrate (600 mg/kg) and perfused transcardially with cold saline followed with 4% paraformaldehyde, then the brains were removed and fixed in the same fixative solution for 2 h. Slices were embedded in paraffin and cut into 6- μ m-thick coronal sections. The rats for RT-PCR examination were decapitated quickly, then the

right superior one-third of cortical areas from the longitudinal cerebral fissure to lateral cerebral fissure, which was defined as penumbra area^[8], were dissected and stored at -70 °C until use.

2.3 Immunohistochemical staining The sections were dropped with 3% H_2O_2 for 10 min at 37 °C, washed three times in distilled water for 3 min each, then heated and boiled by microwave. Nonspecific activity was blocked with protease for 20 min. A rat polyclonal anti-caspase-12 antibody (1:160, Chemicon Ltd. USA) was incubated with the brain section at 4 °C overnight, washed in PBS for three times (2 min each) followed by incubated with the secondary antibody (biotin-labelled goat anti-rabbit IgG) at 37 °C for 40 min, and then washed in PBS for three times (2 min each) again. Finally, the sections were stained with DAB and counterstained with hematoxylin for 15 min. Caspase-12 positive cells were labeled brown in the cytoplasm.

2.4 RT-PCR The primers were synthesized as follows: caspase-12 (GenBank NM-130422), forward 5'-TCC TGG TCT TTATGTCCC-3', reverse 5'-CGATAGCCCAAGGAAGTG-3', with a 180-bp product; and the internal control β -actin: forward 5'-CAC CCT GTG CTG CTC ACC GAG GCC-3', reverse 5'-CCA CAC AGA TGA CTT GCG CTC AGG-3', with a 690-bp product. The PCR reaction system (25 μ L) contained ddH₂O 17.1 μ L, cDNA fragment 3.0 μ L, 10 $\times$ Buffer 2.5 μ L, dNTPs (2.5 mmol/L) 2 μ L, Taq polymerase 0.2 μ L, and primers for caspase-12 or β -actin 0.1 μ L. PCR reaction parameters were as follows: pre-degeneration at 94 °C for 10 min, degeneration at 94 °C for 40 s, annealing at 51.5 °C for 1 min, and elongation at 72 °C for 1 min, for 32 cycles; and finally a continual elongation at 72 °C for 7 min. The kit was supplied by Dalian Bao Biological Technology Ltd., China.

2.5 Statistical analysis Under four unoverlapped visual fields, the integral optical density (IOD) of caspase-12 protein was calculated by using MetaMorph image analysis system. PCR products were detected by 2% agarose gel electrophoresis and analyzed by 1D Kodak image analysis system. The relative quantity of caspase-12 mRNA was estimated by comparison of its absorbance ratio to that of β -actin's. Data were analyzed by one-way ANOVA with SPSS11.5 software package and expressed as mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Expressions of caspase-12 protein Caspase-12 protein were mainly expressed in the ischemic penumbra. There were no caspase-12 positive cells observed in sham operation group. The caspase-12 protein level in the other three groups was significantly higher than that in sham operation group ($P < 0.01$). The caspase-12 level began to increase at 6 h after the ischemia-reperfusion, gradually increased further after that until reached peak at 24 h, and then decreased gradually. The expression of caspase-12 protein in the K_{ATP} opener group were significantly less than that in the ischemia-reperfusion group and the K_{ATP} blocker group at each time ($P < 0.01$, $P < 0.05$). There were no significant differences between ischemia-reperfusion group and K_{ATP} blocker group

Fig. 1 Comparison of caspase-12 protein levels among different groups. There were no caspase-12 positive cells observed in sham operation group. * $P < 0.05$, ** $P < 0.01$ vs ischemia-reperfusion group; # $P < 0.05$, ## $P < 0.01$ vs K_{ATP} opener group.

Fig. 2 Expression of caspase-12 protein at 24 h after ischemia-reperfusion in different groups detected by immunohistochemistry. Caspase-12 positive cells were labeled brown in the cytoplasm. A: Sham operation group; B: ischemia-reperfusion group; C: K_{ATP} opener group; D: K_{ATP} blocker group. Scale bar, 20 μ m.

at each time ($P > 0.05$). (Fig. 1 and Fig. 2)

3.2 Expressions of caspase-12 mRNA The caspase-12 mRNA was expressed weakly in sham operation group. In the ischemia-reperfusion group, the level of caspase-12 mRNA increased at 6 h after ischemia-reperfusion compared with the sham operation group ($P < 0.01$), reached peak at 24 h,

and then decreased gradually. The expression of caspase-12 mRNA in the K_{ATP} opener group at each time were significantly less than that in the ischemia-reperfusion group and the K_{ATP} blocker group ($P < 0.01$). There were no differences between ischemia-reperfusion group and K_{ATP} blocker group at each time ($P > 0.05$) (Fig. 3 and Fig. 4).

Fig. 3 Comparison of caspase-12 mRNA among different groups. * $P < 0.01$ vs sham operation group; ** $P < 0.01$ vs ischemia-reperfusion group; & $P < 0.01$ vs K_{ATP} opener group.

Fig. 4 Expression of caspase-12 mRNA at 24 h after ischemia-reperfusion in different groups. M: marker; A: sham operation group; B: ischemia-reperfusion group; C: K_{ATP} opener group; D: K_{ATP} blocker group.

4 Discussion

K_{ATP} channels are composed of heteromultimers: four inward rectifier potassium channel pore subunits (Kir6.X) and four sulfonylurea receptors (SURs) that regulate channel activity. K_{ATP} channels are distributed widely in the central nervous system. K_{ATP} channel opener is a new neuroprotective drug, but its protective mechanism is still elusive. Our previous studies showed that K_{ATP} channel opener significantly decreased the infarct volumes, improved neurological deficits, inhibited neuronal apoptosis, decreased the expression of caspase-3, caspase-8, caspase-9 mRNAs and proteins by regulating both death receptor and mitochondria signal pathways^[3,4].

ER stress pathway is a new signal pathway different from death receptor and mitochondria signal pathways. ER

distribute widely in eukaryotic cells. It is an important organelle involved in folding and processing of proteins, lipids metabolism, and calcium store^[9]. The activation of caspase-12 is a key factor in ER stress-induced apoptosis. Caspase-12 in the ER is synthesized as an inactive proenzyme procaspase-12, which converted into the active enzyme responding to ER stress. Active caspase-12 then activates subsequent caspases such as caspase-9 and caspase-3, and induced neuronal apoptosis^[10]. By immunohistological analysis, Shibata M *et al.*^[11] demonstrated that caspase-12 was activated in neurons at 5-23 h after transient MCAO, and caspase-12 immunoreactivity was co-localized with neuronal apoptosis. Mouw G *et al.*^[12] reported that caspase-12 was also activated after permanent focal ischemia in rats. We observed that the expressions of caspase-12 mRNA and protein were significantly elevated after cerebral ischemia by immunohistological and RT-PCR analysis. The number of caspase-12 positive cells began to increased at 6 h, increased further after that until reached peak at 24 h, and then decreased gradually. This dynamic change was consistent with neuronal apoptosis. The results indicated that ER stress pathway took part in regulation of the neuronal apoptosis following the cerebral ischemia-reperfusion. After cerebral ischemia-reperfusion, ER stress can be induced under several conditions like hypoxia, acidosis, ATP depletion, perturbation of intracellular calcium homeostasis, and excess free radical production. The moderate stress on ER will trigger many rescuer responses; on the other hand, excessive or long-term exposure to ER stress will induce tissue damages. Following disruption of the calcium homeostasis in ER, m-calpain is activated. Procaspase-12 is converted into active enzyme following cleavage of activated m-calpain, and finally leads to activation of the downstream caspases^[13]. ER is also susceptible to oxidative stresses. Cerebral ischemia-reperfusion injuries can trigger the generation of intracellular reactive oxygen species, which will lead to ER oxidative damages^[14].

Whether K_{ATP} opener has an effect on ER stress pathway was not reported before. Our study indicated that K_{ATP} opener pinacidil could significantly reduce the expressions of caspase-12 mRNA and protein following cerebral ischemia-reperfusion. The protective effect of pinacidil could be completely abolished by K_{ATP} blocker glibenclamide. This result demonstrated that K_{ATP} opener protected neuronal cells

against neuronal apoptosis following the cerebral ischemia-reperfusion by intervening ER stress pathway. The open of K_{ATP} channel resulted in massive depolarization of the neuronal membrane, close of voltage-dependent calcium channel, decrease of Ca^{2+} influx from the extracellular space and inhibition of intracellular Ca^{2+} release from ER, therefore reduced intracellular Ca^{2+} overload^[15]. We proposed that K_{ATP} opener could reduce the disturbance of calcium homeostasis in ER, by which inhibit ER stress, down-regulate the expression of caspase-12 mRNA and protein, and inhibit the neuronal apoptosis after cerebral ischemia. Furthermore, there were many genes and proteins involved in regulation of apoptosis-associated signal pathway. The different signal pathways are not separated completely but cross into a complex network. It is still uncertain if K_{ATP} opener directly activated ER stress pathway or indirectly activated ER stress pathway by activating other signal pathways.

The present study indicated that ER stress pathway may take part in regulation of the neuronal apoptosis after cerebral ischemia. K_{ATP} opener had an important protective effect against neuronal apoptosis following cerebral ischemia-reperfusion by down-regulating the expressions of caspase-12 mRNA and protein, and inhibiting the ER stress pathway. ER plays a potential role in many physiological or pathological processes. Therefore, as an axis for signal reception, integration and transduction, ER will be a potentially therapeutic target for ischemic stroke.

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ATP 敏感性钾通道开放剂对大鼠脑缺血再灌注后 caspase-12 mRNA 及蛋白表达的影响

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摘要: 目的 观察ATP敏感性钾通道(K_{ATP})开放剂对大鼠脑缺血再灌注后 caspase-12 mRNA 和蛋白表达的影响, 探讨内质网信号通路是否参与了 K_{ATP} 开放剂对脑缺血后神经元凋亡的抑制机制。**方法** 200只Wistar雄性大鼠随机分为假手术组, 缺血再灌注组, 开放剂组及阻断剂组。应用线栓法制备大鼠大脑中动脉缺血再灌注模型, 分别应用免疫组化染色、RT-PCR 技术检测脑缺血再灌注后各组 caspase-12 蛋白及 mRNA 的表达。**结果** 在缺血再灌注组, 开放剂组及阻断剂组, 随着缺血再灌注时间的延长, caspase-12 mRNA 及蛋白的表达逐渐增高, 在缺血再灌注后 24 h 达高峰。开放剂组 caspase-12 mRNA 及蛋白表达在各时间点均显著少于缺血再灌注组及阻断剂组 ($P < 0.05$ 或 $P < 0.01$)。阻断剂组各时间点与缺血再灌注组相比均无显著性差异 ($P > 0.05$)。**结论** K_{ATP} 开放剂可能通过抑制内质网信号通路, 减少神经元凋亡, 降低脑缺血再灌注损伤。

关键词: ATP 敏感性钾通道开放剂; 脑缺血; 凋亡; 内质网; Caspase-12