Pinacidil reduces neuronal apoptosis following cerebral ischemia-reperfusion in rats through both mitochondrial and death-receptor signal pathways

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Abstract: Objective To investigate effect of pinacidil, an ATP sensitive potassium channel (K_{ATP}) opener, on the neuronal apoptosis and its signaling transduction mechanism following focal cerebral ischemia-reperfusion in rats. **Methods** One hundred male Wistar rats were randomly divided into four groups: A, sham-operated group; B, ischemia-reperfusion group; C, K_{ATP} opener treatment group; and D, K_{ATP} opener and blocker treatment group. The middle cerebral artery occlusion (MCAO) model was established by using the intraluminal suture occlusion method, neuronal apoptosis was determined by TUNEL staining, and expressions of caspase-8, caspase-9 and caspase-3 mRNA were detected by *in situ* hybridization. **Results** (1) The numbers of apoptotic neurons at 12 h, 24 h, 48 h, and 72 h were significantly less in group C than in groups B and D ($P < 0.01$ or $P < 0.05$); and there was no difference between groups B and D at all time points ($P > 0.05$). (2) The expressions of caspase-3 mRNA and caspase-8 mRNA at all times and the expressions of caspase-9 mRNA at 12 h, 24 h, 48 h, 72 h were significantly lower in group C than in groups B and D ($P < 0.01$ or $P < 0.05$); and there were no differences between groups B and D at all time points ($P > 0.05$). **Conclusions** K_{ATP} opener can significantly decrease the neuronal apoptosis and the expressions of caspase-3, caspase-8 and caspase-9 mRNAs following cerebral ischemia-reperfusion. The neuronal apoptosis may be decreased by the inhibition of both mitochondrial and death-receptor signal pathways.

Keywords: pinacidil; glipizide; cerebral ischemia; apoptosis; mitochondria; death-receptors; signal pathway; caspase-3; caspase-8; caspase-9

1 Introduction

Neurons subjected to ischemia may undergo necrosis or apoptosis^[1,2]. Recent work has shown that neuronal apoptosis occurs primarily within the ischemic penumbra^[3]. It is recognized that both mitochondrial and death-receptor signal pathways are involved in the transduction of apoptotic signals during cerebral ischemia^[4]. The mitochondrial signal pathway is initiated by the release of cytochrome C, which then activates caspase-9. While the death-receptor signal pathway is initiated by the binding of death-

receptors with their ligands, which then induces the activation of caspases-8. Therefore mitochondrial and death-receptor signal pathways are separately characterized by the activation of caspase-9 and caspase-8, both of which can activate the down-stream caspase-3 and then induce the apoptosis^[5].

Recent studies have shown that ATP-sensitive potassium channel (K_{ATP}) has the neuroprotective effect against the apoptosis induced by cerebral ischemia^[6], and so attracts considerable interests for its protection of the ischemic brain. Though K_{ATP} openers can ameliorate neuronal apoptosis, the mechanism is still obscure. In the present study, we examined the effect of pinacidil, a K_{ATP} opener, on the neuronal apoptosis and determined expressions of caspase-3, caspase-8 and caspase-9 mRNAs by using the terminal deoxynucleotidyl transferase-mediated

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dUTP nick end labeling (TUNEL) assay and *in situ* hybridization, respectively, to study the signal transduction pathways of K_{ATP} opener after cerebral ischemia-reperfusion.

2 Materials and methods

2.1 Experimental animals and grouping One hundred male Wistar rats weighing 250-300 g (provided by the laboratory animal center of China Medical University) were randomly assigned into four groups: A, sham-operated group; B, ischemia-reperfusion group; C, K_{ATP} opener treatment group; and D, K_{ATP} opener and blocker treatment group. In each group, the rats were separated into five subgroups ($n = 5$ for each subgroup), in accordance to 6 h, 12 h, 24 h, 48 h, and 72 h after ischemia-reperfusion.

2.2 Preparation of middle cerebral artery occlusion (MCAO) model The MCAO model was performed as Nagasawa *et al.* described^[7]. In brief, the right common carotid artery (CCA) was exposed through a midline incision on the neck and dissected from the 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 a point about 18 mm distal to the carotid bifurcation until mild resistance was indicated. Reperfusion was accomplished by withdrawing the filament at 2 h after MCAO. The operating procedures of sham-operated group were the same to the other three groups except for without inserting a nylon filament. Body temperature was controlled at 37.0 °C with a heating lamp.

2.3 Chemical administration Chemicals were administered referring to the works of Heurteaux C *et al.*^[8]. In group C, 10 μ L of pinacidil (the K_{ATP} opener, 10 nmol/ 5 μ L) was administered intracerebroventricularly to the rats 30 min before induction of cerebral ischemia. In group D, 10 μ L of glipizide (the K_{ATP} blocker, 1 μ mol/ 5 μ L) was injected intracerebroventricularly 15 min before pinacidil application. The rats in groups A and B only received the same volume of 0.9% NaCl (i.c.v.).

2.4 Preparation of brain sections The animals were anesthetized with 10% chloral hydrate (600 mg/kg) and perfused transcardially with cold saline followed by 4% paraformaldehyde. The brains were removed and fixed in the same fixative solution for 2 h. Then samples were embedded in paraffin and cut into 6 μ m-thick coronal sections.

2.5 TUNEL assay The kit was supplied by Wuhan Boster Biological Technology Ltd., China. Deparaffined slides were

treated with 3% H_2O_2 for 10 min at room temperature. After washing three times in distilled water for 2 min each, the slides were dealt with protease K for 10 min at 37 °C. Each slide was labeled with 20 μ L of label liquid for 2 h at 37 °C, then dealt with 50 μ L of enclosed liquid for 30 min at room temperature, and subsequently was dropped with 50 μ L of biotin-labeled digoxin antibody for 30 min at 37 °C. Finally, the slides were stained with diaminobenzidine (DAB), and counterstained with hematoxylin. The apoptotic cells were labeled brown in nuclei.

2.6 In situ hybridization The kit was supplied by Wuhan Boster Biological Technology Ltd., China. Deparaffined slides were treated with 3% H_2O_2 for 20 min at room temperature, and washed three times in distilled water for 3 min each, then digested with trypsin for 10 s. After washing three times in PBS (pH 7.6) for 5 min each, the slide was incubated with 20 μ L of pre-hybridization solution at 37 °C for 4 h, followed by incubated with 20 μ L of probe-containing hybridization solution at 37 °C overnight. The slides were then washed sequentially in $2 \times$ SSC (twice for 5 min each), $0.5 \times$ SSC and $0.2 \times$ SSC (for 15 min each) to remove the unbound probes. Next, the slides were blocked at 37 °C for 30 min and mixed with anti-DIG-biotin at 37 °C for 1 h, then washed four times in PBS (pH 7.6) for 5 min each. Labeled mRNA was visualized by streptavidin-biotin complex diaminobenzidine (SABC-DAB) staining. The slides were incubated with SABC at 37 °C for 20 min and thereafter with biotinylated-peroxidase for 20 min, then washed four times in PBS (pH 7.6) for 5 min each. Peroxidase activity was visualized by a color reaction with DAB. Finally, sections were counterstained with hematoxylin. Positive cells were labeled brown in cytoplasm.

2.7 Statistical analysis Under four unoverlapped visual fields, the numbers of apoptotic cells and the integral optical density (IOD) of caspase-3, caspase-8, and caspase-9 mRNAs were calculated by using MetaMorph image analysis system (UIC, USA). All data were expressed as mean $\pm$ SD and analyzed by using one-way ANOVA with SPSS11.0 software package. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Neuronal apoptosis The apoptotic neurons were mainly located around ischemic tissues, that was, ischemic penumbra, but rare in the ischemic core region. There were only a few apoptotic cells in the group A, much less than those in B, C and D ($P < 0.05$ or $P < 0.01$). In groups B, C and

D, the number of apoptotic cells began to increase at 6 h after cerebral ischemia-reperfusion, reached the peak at 24 h, and then decreased gradually. The number of apoptotic cells in group C was not significantly different from that in group B or D ($P > 0.05$) at 6 h after ischemia-reperfusion, but was significantly less than that at other time points ($P < 0.01$ or $P < 0.05$). There was no difference between groups B and D at any time point ($P > 0.05$) (Tab. 1, Fig. 1).

3.2 Expressions of caspase-3, caspase-8 and caspase-9 mRNAs There were only a few caspase-3, caspase-8 or caspase-9 positive cells in group A, much less than the other three groups ($P < 0.05$ or $P < 0.01$). The positive

cells in groups B, C and D are mainly located in the ischemic penumbra. The numbers of positive cells began to increase at 6 h after the ischemia-reperfusion, reached the peak at 24 h, then decreased gradually. The expression of caspase-9 mRNA in group C at 6 h was not significantly different from that in groups B or D ($P > 0.05$). The expressions of caspase-9 at other times and caspase-8 and caspase-3 at all time points in group C were significantly lower than those in groups B and D correspondingly ($P < 0.01$, or $P < 0.05$). There was no difference between groups B and D at any time point ($P > 0.05$) (Tab. 2-4, Fig. 2).

Tab. 1 Comparison of apoptotic cells in different groups (mean $\pm$ SD)

Groups	<i>n</i>	Subgroups				
		6 h	12 h	24 h	48 h	72 h
A (sham-operated)	5	1.05 $\pm$ 0.65	0.95 $\pm$ 0.60	1.05 $\pm$ 0.48	1.05 $\pm$ 0.57	0.95 $\pm$ 0.60
B (ischemia-reperfusion)	5	14.45 $\pm$ 6.04**	21.70 $\pm$ 7.93**	44.50 $\pm$ 15.97**	30.19 $\pm$ 6.46**	21.95 $\pm$ 5.23**
C (K _{ATP} opener)	5	10.55 $\pm$ 5.15*	12.35 $\pm$ 5.25**#&	22.65 $\pm$ 9.21**#&&	19.05 $\pm$ 4.80**#&&	13.90 $\pm$ 4.93**#&
D (K _{ATP} opener and blocker)	5	13.05 $\pm$ 7.05**	20.65 $\pm$ 7.14**	44.05 $\pm$ 13.54**	31.45 $\pm$ 8.29**	22.30 $\pm$ 6.26**

* $P < 0.05$, ** $P < 0.01$ vs group A; # $P < 0.05$, ## $P < 0.01$ vs group B; & $P < 0.05$, && $P < 0.01$ vs group D

Tab. 2 Comparison of caspase-3 mRNA in different groups (mean $\pm$ SD)

Groups	<i>n</i>	Subgroups				
		6 h	12 h	24 h	48 h	72 h
A (sham-operated)	5	2.11 $\pm$ 0.88	2.07 $\pm$ 0.83	1.94 $\pm$ 0.72	1.72 $\pm$ 0.86	1.23 $\pm$ 0.63
B (ischemia-reperfusion)	5	15.16 $\pm$ 5.49**	26.22 $\pm$ 8.99**	36.58 $\pm$ 11.02**	28.05 $\pm$ 10.03**	20.32 $\pm$ 7.26**
C (K _{ATP} opener)	5	8.16 $\pm$ 3.52*#&	15.49 $\pm$ 5.69**#&	21.27 $\pm$ 5.98**#&&	14.34 $\pm$ 6.96*#&	11.99 $\pm$ 4.72**#&
D (K _{ATP} opener and blocker)	5	15.45 $\pm$ 5.23**	24.75 $\pm$ 8.39**	37.44 $\pm$ 10.43**	27.90 $\pm$ 10.90**	13.33 $\pm$ 9.53**

* $P < 0.05$, ** $P < 0.01$ vs group A; # $P < 0.05$, ## $P < 0.01$ vs group B; & $P < 0.05$, && $P < 0.01$ vs group D

Tab. 3 Comparison of caspase-8 mRNA in different groups (mean $\pm$ SD)

Groups	<i>n</i>	Subgroups				
		6 h	12 h	24 h	48 h	72 h
A (sham-operated)	5	1.91 $\pm$ 0.60	1.76 $\pm$ 0.59	1.82 $\pm$ 0.70	1.91 $\pm$ 0.76	1.90 $\pm$ 0.57
B (ischemia-reperfusion)	5	12.33 $\pm$ 4.83**	24.58 $\pm$ 8.83**	34.36 $\pm$ 9.70**	26.58 $\pm$ 8.45**	20.09 $\pm$ 6.98**
C (K _{ATP} opener)	5	7.16 $\pm$ 3.25*#&	14.18 $\pm$ 5.21**#&	20.50 $\pm$ 6.23**#&&	15.96 $\pm$ 4.87**#&	11.48 $\pm$ 4.62**#&
D (K _{ATP} opener and blocker)	5	12.25 $\pm$ 4.65**	23.66 $\pm$ 8.40**	34.94 $\pm$ 9.18**	25.80 $\pm$ 7.19**	19.84 $\pm$ 5.93**

* $P < 0.05$, ** $P < 0.01$ vs group A; # $P < 0.05$, ## $P < 0.01$ vs group B; & $P < 0.05$, && $P < 0.01$ vs group D

Tab. 4 Comparison of caspase-9 mRNA in different groups (mean $\pm$ SD)

Groups	<i>n</i>	Subgroups				
		6 h	12 h	24 h	48 h	72 h
A (sham-operated)	5	1.60 $\pm$ 0.70	1.74 $\pm$ 0.52	1.82 $\pm$ 0.69	1.95 $\pm$ 0.83	1.82 $\pm$ 0.76
B (ischemia-reperfusion)	5	10.99 $\pm$ 4.98**	22.21 $\pm$ 8.62**	33.06 $\pm$ 12.12**	27.44 $\pm$ 8.47**	19.42 $\pm$ 5.74**
C (K _{ATP} opener)	5	7.42 $\pm$ 3.54*	12.64 $\pm$ 6.44*#&	20.04 $\pm$ 8.25**#&	17.42 $\pm$ 6.10**#&	10.94 $\pm$ 3.67**#&
D (K _{ATP} opener and blocker)	5	11.13 $\pm$ 4.48**	22.61 $\pm$ 9.01**	32.43 $\pm$ 11.13**	26.45 $\pm$ 8.17**	19.14 $\pm$ 6.56**

* $P < 0.05$, ** $P < 0.01$ vs group A; # $P < 0.05$ vs group B; & $P < 0.05$ vs group D

Fig. 1 Neuronal apoptosis in different groups at 24 h after ischemia-reperfusion (TUNEL assay). A: Sham-operated group; B: ischemia-reperfusion group; C: K_{ATP} opener treatment group; D: K_{ATP} opener and blocker treatment group. Arrows indicate the apoptotic cells. Scale bar, 20 μ m.

Fig. 2 Expressions of caspase-3, caspase-8, and caspase-9 mRNAs in different groups at 24 h after ischemia-reperfusion (*In situ* hybridization). A: sham-operated group; B: ischemia-reperfusion group; C: K_{ATP} opener treatment group; D: K_{ATP} opener and blocker treatment group. In each column, there were a few caspase-3, caspase-8 or caspase-9 positive cells in group A, positive cells were significantly lower in group C than in groups B and D, and there was no difference between groups B and D. Arrows indicate the caspase-3, caspase-8, and caspase-9 positive cells separately in the corresponding columns. Scale bar, 20 μ m.

4 Discussion

K_{ATP} channels are heteromultimers composed of four inward rectifier potassium channel pore subunits (Kir6.X) and four sulfonylurea receptors (SURs) that regulate channel activity^[9]. There are high density K_{ATP} channels in the substantia nigra, globus pallidus, cerebral cortex, cerebellum and hippocampus of the brain. One of the important properties of K_{ATP} channels is that once opened, they can be blocked by sulfonylureas such as glipizide. The neuroprotective role of K_{ATP} channels opener has been reported in *in vitro* and *in vivo* hypoxia and ischemia models. Teshima Y *et al.*^[10] reported that the activated mitochondrial K_{ATP} channels could protect cerebellar granule neurons from the apoptosis induced by oxidative stress. Our results demonstrated that the K_{ATP} channels activated by pinacidil could significantly reduce neuronal apoptosis after cerebral ischemia, and the protective effect of pinacidil could be ameliorated by K_{ATP} blocker glipizide. The results were consistent with previous studies.

The signal transduction mechanism of K_{ATP} opener in inhibiting neuronal apoptosis is still unclear. Two main signal pathways identified in regulating neuronal apoptosis are the mitochondrial signal pathway and the death-receptor signal pathway^[11]. The mitochondrial signal pathway is initiated by the release of pro-apoptotic intramitochondrial factors such as cytochrome C. In cytosol, Apaf-1 couples cytochrome C to form a multiproteic complex, known as the apoptosome. Interactions between cytochrome C and Apaf-1 in the apoptosome promote the recruitment of pro-caspase-9 and the auto-conversion of pro-caspase-9 into active caspase-9. Thus caspase-9 cleaves effector caspases, such as caspase-3, to lead the affected cell to the point-of-no-return of the death process. Death signals provided by the environment are integrated by a kind of receptors belonging to the tumor necrosis factor (TNF) receptor superfamily, the so called death-receptors. Death-receptors are characterized by the presence of an intracytoplasmic death domain (DD), mainly including TNF-R1, Fas, DR3, DR4, and DR5. Binding to their ligands induces the trimerization of the receptors, and DD of which would interact with the adaptor proteins containing a death effector domain (DED). A multiproteic complex is thus formed known as the death-inducing signal complex (DISC), which can promotes the recruitment and activation of initiator procaspases-8. Activated caspase-8 propagates the apoptotic signal by directly activating downstream effector caspases

such as caspase-3^[12]. Therefore, we can study the death-receptor and mitochondrial signal pathway by detecting changes of caspase-8 and caspase-9 mRNAs.

In the present results, mRNAs of caspase-3, caspase-8 and caspase-9 after cerebral ischemia were all overexpressed, indicating that both the mitochondrial and death-receptor signal pathways take part in the regulation of neuronal apoptosis. Intracerebroventricular injection of K_{ATP} opener pinacidil could significantly reduce the expressions of caspase-8 and caspase-9 mRNAs, and this effect could be ameliorated by K_{ATP} blocker glipizide. The results suggest that K_{ATP} opener may exert the protective effect against the neuronal apoptosis induced by cerebral ischemia-reperfusion by inhibiting both the death-receptor and mitochondrial signal pathways.

Many reports showed that K_{ATP} opener can regulate expressions of many genes related to apoptosis. Cheng HX *et al.* reported that K_{ATP} opener could inhibit cardiomyocyte apoptosis and down-regulate the protein expression of Fas gene during myocardial ischemia-reperfusion in rats^[13]. Busija DW *et al.* reported that K_{ATP} opener attenuated Ca²⁺ accumulation in mitochondria, prevented the permeability transition pore (PTP) opening and the mitochondrial inner membrane potential ($\Delta\Psi_m$) loss, and reduced the release of cytochrome C from mitochondria^[14]. The two signal pathways were not separated but crossed. In death-receptor signal pathway, activated caspase-8 can directly activate down-stream caspase-3 and also cleave Bid into truncated Bid (tBid) at the same time, which translocates to the mitochondria and induces first the clustering of mitochondria around the nuclei and the release of cytochrome C. Therefore, tBid is a mediator between mitochondrial signal pathway and death-receptor signal pathway^[15].

In conclusion, we have provided evidence to indicate that K_{ATP} opener has protective effect against neuronal apoptosis after cerebral ischemia-reperfusion by inhibiting both death-receptor and mitochondrial signal pathways. K_{ATP} channels could be a possible target for neuroprotective mechanism. Further studies are required to explore the more detailed mechanisms of the K_{ATP} opener's protective effects.

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Pinacidil 抑制线粒体和死亡受体通路减少大鼠脑缺血再灌注后神经元凋亡

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摘要: 目的 探讨ATP敏感性钾通道开放剂pinacidil对大鼠脑缺血再灌注后神经元凋亡的保护作用及信号转导机制。方法 100只Wistar雄性大鼠随机分为四组: A组(假手术组)、B组(缺血组)、C组(K_{ATP} 开放剂处理组)及D组(K_{ATP} 开放剂和阻断剂处理组)。用线栓法制备大鼠脑中动脉缺血(middle cerebral artery occlusion, MCAO)模型, 用DNA断末端标记法(terminal-deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, TUNEL)检测神经元凋亡, 用原位杂交方法检测 caspase-3、caspase-8 及 caspase-9 mRNA 的表达。结果 (1) C组 12 h、24 h、48 h、72 h 时间点的凋亡细胞数较 B、D 组显著减少($P < 0.05$ 或 $P < 0.01$); B 组和 D 组之间无显著性差异($P > 0.05$)。(2) C 组 caspase-3 mRNA 和 caspase-8 mRNA 在各时间点及 caspase-9 mRNA 在 12 h、24 h、48 h、72 h 时间点的表达显著少于 B 组和 D 组($P < 0.01$ 或 $P < 0.05$), B 组和 D 组之间无显著性差异($P > 0.05$)。结论 K_{ATP} 通道开放剂能显著减少大鼠脑缺血再灌注后的细胞凋亡及 caspase-3、caspase-8 及 caspase-9 mRNA 的表达。 K_{ATP} 通道开放剂可能通过抑制线粒体通路和死亡受体通路降低神经元凋亡, 保护缺血再灌注损伤后的脑组织。

关键词: pinacidil; glipizide; 脑缺血; 凋亡; ATP 敏感性钾通道; 线粒体; 死亡受体; 信号通路; caspase-3; caspase-8; caspase-9