Protective effects of IGF-1 on neurons under condition of hypoxia and the role of PI3K signal pathway

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Abstract: Objective To investigate the protective effects of insulin like growth factor 1 (IGF-1) on cortical neurons under condition of hypoxia and the possible mechanism. **Methods** Cerebral cortical neurons from newborn rats were cultured under the condition of oxygen and glucose deprivation (OGD). On day 7, neurons were treated with IGF-1 or IGF-1 plus LY294002 or PD98059 under condition of OGD or normal condition. MTT assay was used to analyze the viability of neurons in each group. The expression of total Akt and p-Akt were analyzed by Western blot. **Results** Compared with the control, the neuron viability was significantly higher in IGF-1 treated group under normal or OGD condition ($P < 0.05$). The protective effects of IGF-1 were attenuated in the presence of LY294002 but not PD98059. The result of Western blot showed IGF-1 upregulated the expression of p-Akt, which was inhibited by LY294002. **Conclusion** PI3K pathway may play an important role in neuroprotection afforded by IGF-1.

Key words: insulin like growth factor 1; neuron; signal pathway; oxygen and glucose deprivation model

胰岛素样生长因子 1 对缺血缺氧神经元的保护及其与 PI3K 信号转导通路的关系

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[**摘要**] **目的:** 观察胰岛素样生长因子-1(insulin like growth factor 1, IGF-1)对缺氧缺糖神经元的保护作用并探讨其可能的作用机制。**方法:** 构建体外培养的神经元氧糖剥夺模型(oxygen and glucose deprivation, OGD),第 7 天将培养的神经元分为 8 组(4 组暴露于氧糖剥夺,另 4 组非暴露),分别施加纯化的 IGF-1 单体,并观察加入 PI3K 和 MAPK 信号通路的特异性阻断剂 LY294002 和 PD98059 的效应,利用 MTT 法分别观察各组神经元的细胞活性; Western 印迹观测不同干预因素下 Akt 和 p-Akt 蛋白的表达情况。**结果:** 神经元正常组和缺血缺氧模型组,加入 IGF-1 后细胞增殖活性均显著升高($P < 0.05$);而同时加入 IGF-1 和 LY294002 后,IGF-1 促神经元活性的作用被明显抑制($P < 0.05$),反之同时加入

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IGF-1 与 PD98059 后,IGF-1 发挥促神经元存活的作用未被明显阻滞($P>0.05$)。Western 印迹结果示 IGF-1 可显著上调 p-Akt 的表达,这种上调作用可以被 LY294002 阻滞。**结论**:IGF-1 有明确的神经保护作用,其可能是通过 PI3K/Akt 通路来发挥作用的。

[**关键词**] 胰岛素样生长因子-1; 神经元; 信号通路; 氧糖剥夺模型
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Insulin like growth factors (IGFs) are a group of multifunctional cell-proliferation-regulating factors that are involved in the process of cellular growth, differentiation, apoptosis, and transformation. The IGFs family consists of IGF-1, IGF-2, insulin growth factor 1 receptor (IGF-1R), IGF-2R, and 6 IGF binding proteins (IGFBP1 – 6)[1-2]. As one of the important members of IGFs family, IGF-1 is found to have the effect of neuroprotection in cerebral injury induced by ischemia and anoxia. The mechanism of neuroprotection effect may be that IGF-1 combines with its specific receptor IGF-1R, which activates the corresponding signal pathway and inhibits the apoptosis of nerve cells in the ischemic regions. The process of phosphatidylinositol-3-kinase (PI3K) activating Akt is thought to be the most important way of IGF-1 system accomplishing the function of protecting cells and resisting apoptosis. It is also concluded that the IGF-1-mediated anti-apoptosis function may be realized through several signal pathways including PI3K and mitogen-activated protein kinase (MAPK). In this research, we observed the effect of IGF-1 on neurons in oxygen and glucose deprivation (OGD) environment and explored its possible mechanism by establishing the OGD neuron model, depurating IGF-1 monomer in vitro, and blocking signal pathways of PI3K and MAPK.

1 MATERIALS AND METHODS

1.1 Aanimals, main reagents, and equipments

Five specific-pathogen-free SD neonate rats under 24-hours old were bought from Beijing Vital River Laboratory Animal Technology Co. Ltd. Neural cell basal medium (Neurobasal-A medium) and B27 were purchased from Gibco Company in the USA; poly-*L*-lysine and trypsin were from Sigma Company in the USA; PI3K channel blocker LY294002, MAPK channel blocker PD98059, Akt antibody and phospho-Akt (p-Akt) antibody were from Cell Signaling Company in the USA; fetal calf serum was from Beijing Yuanheng Jinma Biotechnology development Co. Ltd.; methyl thiazolyl tetrazolium (MTT) was from Amresco Company in the USA; 24-well culture plate, 96-well culture plate and 75 cm^2 cell culture flask were from Costar Company in the USA; 0.22 μm filter was from Millipore Company in the USA; thermostatic incubator was from Sanyo Company in Japan; inverted phase contrast microscope was from Nikon Company in Japan; hand-made oxygen-deprivation jar was from Beijing Kepu Instrument Plant.

1.2 Primary culture of cerebral cortex neurons

Poly-*L*-lysine solution were added to each well of the 96-well culture plate (0.1 mL), and the 24-well culture plate (0.5 mL). The plates were shaken slightly so that the solution was in the bottom of the wells. They were placed in room temperature for 20 min and the solution were discarded. Then the plates were washed twice with *D*-Hank solution, ultraviolet-radiated and blow-dried on super clean bench, and sealed for the further use.

The newborn SD rats were pre-frozen at −20 ℃ for 20 min, then their heads were cut down and brains were taken out in aseptic conditions and put into culture dishes with dissection solution. Under the dissecting microscope, the piamater, vessels, and medulla were eliminated, and the cerebral cortex was collected, cut up and put into a 15 mL centrifuge tube. Five milliliter 0.125% trypsin solution were added into the tube to digest the tissue at

37 ℃ for 20 min, and the tube was shaken once or twice during the process. Then the trypsin solution was carefully absorbed, and neurobasal which contained 10% fetal calf serum was added to terminate the digestion. The centrifuge tube was hold still so that the tissue sunk, and the supernate was removed. After the neuron medium containing 5% fetal calf serum, 2% B27, and 1% Glutamax was added to the tube, the liquid was blown lightly with thin straw again and again until it became well-mixed. The suspension was filtered with a 74 μm-pore-diameter filter screen to eliminate undigested tissue pieces, and the filtered single cell suspension was poured into a beaker. The cells were counted with a blood cell counter and the cell density was adjusted to 1.0×10^6/mL. The cells were inoculated into plates custodited with poly-*L*-lysine (100 mL/well for the 96-well culture plate, and 500 μL/well for the 24-well culture plate) at 37 ℃, 5% CO_2 for 24 h. Then the medium was changed into serum-free medium containing neurobasal, 2% B27 and 1% Glutamax. After that, half of the medium was changed and the cells were observed with inverted phase contrast microscope every other days. Neurons incubated for 7 days were used in the experiments.

1.3 Grouping and modeling of neurons

According to related documents[3] and our trials, we determined that the effect concentration of IGF-1 was 0.1 ng/L and that of the blocking agent LY294002 and PD98059 was 10 μmol/L. Neurons that had been incubated for 7 days were divided into 8 groups: a normal group (no IGF-1 was added and the medium was changed into DMEM/F12 when modeling); a IGF-1 group (the medium was changed into DMEM/F12 that contained 0.1 ng/L IGF-1); a LY294002 group (the medium was changed into DMEM/F12 that contained 10 μmol/L LY294002); a PD98059 group (the medium was changed into DMEM/F12 that contained 10 μmol/L PD98059); an OGD group (the basal medium was changed into carbohydrate-free Kreb's solution); an OGD + IGF-1 group (DMEM/F12 containing 0.1 ng/L IGF-1 was added and reacted for 2 h, and then the medium was changed into Kreb's solution containing 0.1 ng/L IGF-1); an OGD + IGF-1 + LY294002 group (DMEM/F12 containing 10 μmol/L LY294002 was added and reacted for 1 h, then 0.1 ng/L IGF-1 was added and reacted for 2 h. After that, the medium was changed into Kreb's solution that contained 0.1 ng/L IGF-1 and 10 μmol/L LY294002); an OGD + IGF-1 + PD98059 group (DMEM/F12 containing 10 μmol/L PD98059 was added and reacted for 1 h, then 0.1 ng/L IGF-1 was added and reacted for 2 h. After that, the medium was changed into Kreb's solution that contains 0.1 ng/L IGF-1 and 10 μmol/L PD98059). The neurons in the former 4 groups were under normal condition, and those in the latter 4 groups were under condition of OGD (placed into an oxygen-deprived jar containing 93% N_2 and 7% CO_2 to establish models for 4 h).

The primary media containing IGF-1 and various blockers in the latter 4 groups were retained and used to replace the Kreb's solution of their corresponding groups after the modeling. And then they were put into the incubator for another 12 h. Cells used for Western blot were collected immediately after the modeling for protein extraction.

1.4 MTT assay to test cell viability

The supernate in the wells was removed, and 100 μL DMEM/F12 and 20 μL MTT (5 mg/mL) solution was added into each well and mixed, then they were placed into a 37 ℃ incubator for 4 h. After the incubation, the supernate was carefully absorbed and removed, and 100 μL DMSO was added into each well, and the culture plate was shaked for 10 min. The OD value of each well was measured at the wavelength of 570 nm, and the reference wavelength was 620 nm.

1.5 Detecting the expression of total Akt and p-Akt by Western blot

After the modeling, the cells were collected and placed on ice. A mixture of RIPA and PMSF

(9∶1) was prepared, and 100 μL mixture was added into each well. Then the total protein was extracted, and its concentration was determined by using of BCA protein assay kit. After that, 50 μg protein was taken from each group for SDS-PAGE, and then it was transferred, blocked with 5% BSA for 1 h, added primary antibodies (Akt antibody or p-Akt antibody), and incubated at 4℃ in swing bed for the night. After being washed, the protein was added with secondary antibody that was marked by HRP and incubated at room temperature in swing bed for 1 h. Then the PVDF membrane was processed with SuperECL Plus chemoluminescence reagent, and exposed in darkroom. After being developed and fixed, specific bands appeared.

1.6 Statistical analysis

All the data were presented with mean ± standard deviation ($\bar{x} \pm s$) and processed via software SPSS14.0. ANOVA and LSD-t were adopted among multi-group comparisons. $P < 0.05$ was considered as statistically significant.

2 RESULTS

2.1 Changes of the cell morphology

Neurons that had cultured for 7 day were plump with the cone, ovum or fusiform shape. Their neurite grew smooth, thin, and long. Obvious halation was found around the cells, and some nucleolus were seen. Neurons in the oxygen-glucose deprivation groups decreased, their bodies swelled to balls, synapses became short and thick, and their refractivity weakened.

2.2 Comparison of proliferative activity among groups

The cell viability of the LY294002 group and PD98059 group did not change significantly compared with the normal group ($P > 0.05$), while the cell viability of the IGF-1 group significantly increased compared with the normal group ($P = 0.007$).

The cell viability of the OGD group significantly decreased compared with the normal group ($P < 0.01$), indicating that the modeling was successful. The viability of neurons that were added with IGF-1 was obviously improved compared with the OGD group ($P < 0.001$). In the OGD + IGF-1 + LY294002 group, IGF-1's effect on the proliferative activity of neurons was evidently inhibited, and the cell viability of those groups was of no obvious difference from that of the OGD group ($P = 0.667$). In the OGD + IGF-1 + PD98059 group, the cell viability was significantly increased compared with the OGD group ($P < 0.001$), indicating that PD98059 did not influence IGF-1's effect on enhancing the viability of neurons (Tab.1).

Tab.1 Viability of neurons in each group ($\bar{x} \pm s$, $n = 16$)

Groups	OD values	P
Normal group	0.218 ± 0.0170	
IGF-1 group	0.245 ± 0.0348##	0.007
LY294002 group	0.201 ± 0.0279	0.080
PD98059 group	0.202 ± 0.0271	0.098
OGD group	0.159 ± 0.0237##	<0.001
OGD + IGF-1 group	0.194 ± 0.0265**	<0.001
OGD + IGF-1 + LY294002 group	0.155 ± 0.0168	0.667
OGD + IGF-1 + PD98059 group	0.187 ± 0.0207**	<0.001

Compared with the normal group, ##$P < 0.01$; compared with the OGD group, **$P < 0.01$.

2.3 Results of Western blot

Akt expression in all groups showed no evident difference. IGF-1 activated the expression of p-Akt, while LY29002 reduced it (Fig.1).

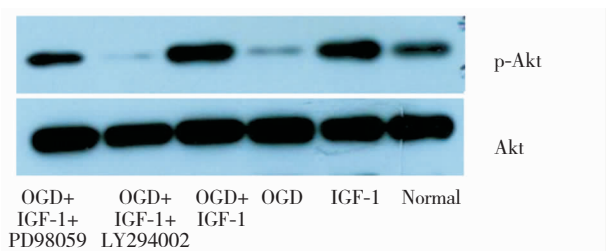

Fig.1 Expression of Akt and p-Akt under different conditions

3 DISCUSSION

In past researches, people always focused on IGF-1's effect on growth and development[4-5]. As the researches progress, IGF-1's function in CNS is also known to more and more people[6-8]. It has been proved in recent researches that IGF has definite effect of neuroprotection on blood/oxygen-deprivation induced cerebral injury. Liu, et al.[9] found that the middle cerebral artery occlusion (MCAO) model rats were given 75 mg IGF-1 10 min, 24 h and 48 h after the model was established, the cerebral infarct volume was reduced by 60% compared with the control group, and the nerve function and proprioceptive reflex greatly improved. By giving IGF-I to the noses of fetal rats with blood/oxygen-deprivation induced cerebral injury, Lin, et al.[10] found that IGF-I could decrease neuroethologic injury, reduce the apoptosis of nerve cells and promote the proliferation of neurons and oligodendrocytes after the blood/oxygen-deprivation.

Since IGF-1 is found to have definite effect of neuroprotection on blood/oxygen-deprivation induced cerebral injury, studies on the mechanism are carried out in recent years. In some researches, the process of PI3K activating Akt is thought to be the most important way of IGF-1 system accomplishing the function of protecting cells and resisting apoptosis[11-12]. It is also concluded that the IGF-1-mediated anti-apoptosis function is closely related to several signal pathways including MAPK and GSK-3β[13-15]. In this research, we proved that under the condition of OGD, IGF-1 had definite effect of anti-apoptosis and protection on cerebral cortex neurons cultured in vitro. The viability of neurons which were added with IGF-1 was obviously improved compared with the OGD group; the using of PI3K blocker significantly inhibited neuroprotection afforded by IGF-1, indicating that PI3K pathway played an important role in mediating neuroprotection of IGF-1. In the group that was added with the specific blocker PD98059 of MAPK pathway, the cell viability was significantly higher than that in the OGD group, but was of no obvious difference from that of the IGF-1 + OGD group, indicating that IGF-1's effect on promoting the neuron viability was not inhibit by PD98059. The above results showed that PI3K pathway was an important way for IGF-1 to protect nerves.

Akt is an important serine/threonine protein kinase of PI3K pathway, and p-Akt is its activated form. Activated Akt can cause cell apoptosis by phosphorylating proteins like BAD and FKHRL[16]. Our result by Western blot had that the total Akt expression in all groups had no difference, but the p-Akt expression of neurons in the OGD + IGF-1 group obviously increased, which started the transmission of downstream signals, and finally improved cell viability and resisted apoptosis. On the other hand, the increase of the expression was evidently inhibited by LY294002, which was also in correspondence with MTT's result. However, whether PD98059 was added or not in the OGD + IGF-1 group had no influence on p-Akt expression, which was also in correspondence with MTT's result. All these results confirmed that IGF-1 has definite effect of neuroprotection, and may work by activating PI3K/Akt pathway.

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