Relationship between transmembrane signal transduction pathway and DNA repair and the mechanism after global cerebral ischemia-reperfusion in rats

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Abstract: Objective To investigate the protein levels of phospho-ERK and phospho-APE/Ref-1 in hippocampal neurons after global cerebral ischemia reperfusion in rats, and observe the relationship between transmembrane signal transduction and repair of DNA damage. The role of ERK signal transduction pathway following global cerebral ischemia reperfusion in rats is further discussed. **Methods** Ninety healthy male SD rats were divided into 3 groups randomly: Sham group (S group), Ischemia reperfusion group (IR group) and Pd98059 pretreatment/ischemia reperfusion group (PD group). Global cerebral ischemia reperfusion model was established by four-vessel occlusion (4-VO) method, and reperfusion was performed 5 minutes following ischemia. Protein levels of phospho-ERK and phospho-APE/Ref-1 were detected using immunohistochemical method at 2 h, 6 h, 12 h, 24 h, 48 h and 72 h after reperfusion, and neuron apoptosis was observed by HE and TUNEL staining. **Results** In CA1 region of IR group, TUNEL positive cells began to appear at 6 h after IR, and reached the apex during 24 h to 48 h. However, TUNEL positive was most strongly exhibited in PD group. In IR group, phospho-ERK was obviously detected in CA3 region at 2 h after IR, and its level was gradually decreased from 6 h until totally absent at 48 h. Besides, phospho-ERK expression in PD group was weaker than that in IR group. For phospho-APE/Ref-1, its expression began to appear in CA1 region in IR group at 2 h after IR, with no obvious changes during 2 h to 12 h. Phospho-APE/Ref-1 expression began to decrease at 24 h and this decrease continued thereafter. Expression level of phospho-APE/Ref-1 in PD group was lower than that in IR group. Results showed the concurrence of decreased phospho-ERK expression level and increased neuron apoptosis after cerebral ischemia reperfusion, the former of which was consistent with the decrease of phospho-APE/Ref-1 expression. Also, the greater the inhibition of ERK phosphorylation was, the greater decrease of APE/Ref-1 expression occurred. **Conclusion** Activation of ERK signal transduction pathway increased the expression of phospho-APE/Ref-1, and thus facilitated the repair of DNA damage. So, activation of ERK signal transduction pathway may protect neurons from apoptosis after cerebral ischemia reperfusion.

Keywords: cerebral ischemia/reperfusion injury; ERK; APE/Ref-1; DNA repair; apoptosis

1 Introduction

It's currently considered that cerebral ischemia/reperfusion

injury (CIR injury) results from combinations of complex factors or mechanisms. Mitogen-activated protein kinase (MAPK) family act as important regulatory enzymes that link plasma membrane surface receptors to expressions of determining genes. As a member of MAPK family, the extracellular signal regulated kinase (ERK) has not been fully understood in its role in CIR injury, or in cell apoptosis after CIR^[1], since controversy still exists among a wide range of reports. Apurinic/aprimidimic endonuclease/redox factor 1 (APE/

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Ref-1) is a kind of endonuclease which can protect neurons from apoptosis and participate in DNA repair^[3], and it is closely related with some injury diseases, such as ischemia, ischemia/reperfusion and bleeding. Since the relationship between extracellular signal transduction and intracellular DNA damage repair has been rarely reported, in this present study, the rat model of global cerebral ischemia reperfusion was first constructed. And after interfering ERK signal transduction pathway by specific inhibitor (Pd98059), alterations in expression levels of both phospho-ERK and phospho-APE/Ref-1 were detected. The relationship between expression changes and neuron apoptosis was analyzed, and the effect of ERK signal transduction pathway on CIR injury was further discussed.

2 Materials and methods

2.1 Animals and establishment of CIR rat model Ninety healthy male SD rats (weighing 280-320 g, supplied by Xi'an Experimental Animal Center of Medical College, Xi'an Jiaotong University) were randomized into 3 groups ($n = 30$, respectively), namely, sham group (S group), ischemia reperfusion group (IR group) and Pd98059 pretreatment/ischemia reperfusion group (PD group). Global cerebral ischemia reperfusion model was established by 4-VO ligation method. Surgery was performed under anaesthesia using 1% pentobarbital (40 mg/kg, i.p.). After the body-righting reflex disappeared, rats were fixed in a prone position. The neck skin was medisectioned to reveal the triangle behind neck, and between its lateral and the first transversus process, the two bilateral vertebral arteries were fixed and coagulated by 25 mV electric coagulator. Then the cut was sewed up one layer by another, and body temperature was maintained at 37 °C. After 24 h, two common carotid arteries were separated and occluded in IR and PD groups, while in S group, neither the vertebral arteries were coagulated nor the common carotid arteries were occluded, but rats were injected with normal saline (NS) by vena caudalis (the same volume with that of PD in rats with the same weight in PD group). In IR group, vertebral arteries were separated and coagulated, followed by NS injection after 24 h. Then common carotid arteries were separated and occluded for 5 min, after which occlusion was stopped and blood flow was

recovered. In PD group, same operations were employed except the pd98059 (Cell Signaling Technology company, USA) injection (0.3 mg/kg, based on previous report^[21]) by vena caudalis, instead of NC injection. Successful global cerebral ischemia reperfusion model was verified by identifying certain symptoms, including mydriasis, tachypnea, pale eyeball and lip cyanosis.

2.2 HE and TUNEL staining Five anesthetized animals were perfused with 4% paraformaldehyde at 2 h, 6 h, 12 h, 24 h, 48 h and 72 h after IR. Brain tissues were embedded in paraffin followed by preparations of coronal sections with a thickness of 4 μ m using a microtome (Leica-2045, Germany). After being stained with hematoxylin and eosin (HE), the slices were examined in hippocampal CA1 and CA3 regions using light microscopy (400 $\times$). Neuron apoptosis was detected by TUNEL staining, according to manufacturer's protocols (Biologists Limited company, Germany). Cells whose nuclei displayed obviously yellowish-brown particles or patching were defined as apoptotic cells. For each rat, 2 slices were randomly selected, and in each slice, 5 fields were observed in CA1 and CA3 regions, respectively. Apoptotic neurons and total neurons were both counted, and apoptosis index (AI) was calculated ($AI = \text{number of apoptotic neurons} / \text{number of total neurons} \times 100\%$).

2.3 Immunohistochemical assay Expression levels of phospho-ERK and phospho-APE/Ref-1 were detected by immunohistochemical assay using SP-IHC kit (Zhongshan biotechnology company, Beijing), according to manufacturer's instructions. The same dilution of rabbit anti-IgG was employed as negative control. Briefly, the brain sections were deparaffinized with xylene, followed by rehydration by ethanol with graded concentrations (50-100%, v/v), and then they were incubated with 3% H₂O₂ for 10 min. Slices were washed 3 times, followed by antigen retrieval for 15 min. After being blocked for 1h under room temperature without any wash, slices were then incubated with rabbit monoclonal anti-ERK (Cell Signaling company, USA) or mouse monoclonal anti-APE/Ref-1 (Santa Cruz, USA) at 4 °C overnight. After being washed 3 times, the slices were then incubated with biotin-conjugated anti-IgG at 60 °C for 30 min. After washing, added horseradish conjugated streptavidin peroxidase to the slices

and kept the reactions at 37 °C for 20 min. Finally, slices were washed and color was developed by DAB. Image was analyzed using Leica Qunatimet570 image analyzing system (Leica, Germany). Five fields in CA1 and CA3 regions were selected respectively, and mean gray level was measured to indicate the expression level of phospho-ERK or phospho-APE/Ref-1. There was a negative relationship between mean gray level and the expression level of phospho-ERK or phospho-APE/Ref-1 (the lower of the mean gray level, the higher expression level of phospho-ERK or phospho-APE/Ref-1).

2.4 Statistical analysis Data were presented as mean±SD. Statistical significance was determined by SPSS 10.0 statistics software. Group comparison was performed by one-factor variance analysis. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Results of HE and TUNEL Staining and the apoptosis indices (AIs) in three groups As shown in Fig. 1, hippocampal pyramidal cells in S group lined regularly and showed morphological integrity, while in IR group, the cells were ir-

regularly distributed, and most of them lost normal structures and shrunk with pyknotic nuclei and pycnosis while their interspaces became wider. This problem seemed even more serious in PD group, where cell structures were hard to be identified, and the cells obviously exhibited interstitial edema.

TUNEL Staining revealed a large number of apoptotic cells in both IR and PD groups (Fig. 2). As compared with S group, both IR and PD groups showed increased apoptosis index (AI) in CA1 and CA3 regions at 6 h, 12 h, 24 h, 48 h and 72 h after IR ($P < 0.05$). Besides, AIs in both CA1 and CA3 regions in PD group 6 h, 12 h, 24 h, 48 h and 72 h after IR were all significantly higher than those in IR group ($P < 0.05$), as indicated in Table 1.

3.2 Phospho-ERK expressions in three groups As shown in Table 2, when compared with PD group, both IR and S groups showed increased phospho-ERK expression levels in CA3 region at each time point after IR ($P < 0.05$), however, there was no significant difference between IR and S group in CA3 region ($P > 0.05$). Also, phospho-ERK expression level in PD group in CA3 region at 2 h, 6 h, 12 h and 24 h after

Table 1. The AIs in three groups at each time point after reperfusion in CA1 and CA3 regions

Groups		2 h	6 h	12 h	24 h	48 h	72 h
CA1	S group	0.25±0.50	1.49±0.67	1.51±0.65	1.57±0.78	2.14±0.48	1.65±0.63
	IR group	2.54±0.38	14.42±4.25*	29.24±6.08*	41.32±7.30*	38.94±7.32*	21.36±5.07*
	PD group	5.63±0.84	29.76±5.13**	43.57±7.11**	60.08±7.68**	53.31±7.36**	31.06±6.56**
CA3	S group	0.25±0.50	1.49±0.67	1.51±0.65	1.57±0.78	2.14±0.48	1.65±0.63
	IR group	1.33±0.29	9.05±3.12*	20.57±6.08*	33.75±6.12*	30.13±6.34*	15.03±4.22*
	PD group	4.04±0.70	23.32±6.02**	31.26±6.10**	49.87±7.26**	42.82±7.21**	22.64±6.10**

* $P < 0.05$ vs S group, ** $P < 0.05$ vs IR group, ($n = 30$, mean±SD).

Table 2. The expression levels of phospho-ERK at each time point after reperfusion in both CA1 and CA3 regions in three groups

Groups		2 h	6 h	12 h	24 h	48 h	72 h
CA1	S group	184±21	186±19	186±25	205±21	206±26	207±26
	IR group	210±29 ^Δ	209±24 ^Δ	209±23 ^Δ	209±24	207±21	208±20
	PD group	214±23 ^Δ	213±21 ^Δ	213±24 ^Δ	212±25	215±20	215±24
CA3	S group	188±23*	189±26*	188±25*	190±26*	194±23*	195±23*
	IR group	175±23*	170±28*	186±23*	193±21*	206±19	212±21
	PD group	214±24 [#]	214±26 [#]	213±22 [#]	213±24 [#]	214±25	215±23

* $P < 0.05$ vs PD group, [#] $P < 0.05$ vs IR group, ^Δ $P < 0.05$ vs S group, ($n = 30$, mean±SD).

Fig. 1 HE staining images of hippocampal pyramidal cells in CA1 region. Results shown are the representatives of three animals in each group. A: S group at 48 h; B: IR group at 48 h; C: PD group at 48 h. Scale bar, 20 μ m.

Fig. 2 TUNEL staining images in CA1 region. Results shown are the representatives of three animals in each group. A: S group at 48 h; B: IR group at 48 h; C: PD group at 48 h. Scale bar, 20 μ m.

IR was much lower than that in IR group ($P < 0.05$). In CA1 region, phospho-ERK expression levels in both IR and PD groups at 2 h, 6 h and 12 h after IR were significantly lower than that in S group ($P < 0.05$). Representative immunohistochemical images of phospho-ERK in CA3 region were shown in Fig. 3.

3.2 Phospho-APE/Ref-1 expressions in three groups

As shown in Table 3, expression level of phospho-APE/Ref-1 in PD group was much lower than that in IR group after IR 2 h, 6 h, 12 h and 24 h ($P < 0.05$) and also than that in S group at each point after IR ($P < 0.05$). There was no significant difference between S and IR groups. Representative immunohistochemical images of phospho-APE/Ref-1 in CA1 region were shown in Fig. 4.

Fig. 3 Immunohistochemical images of phospho-ERK in CA3 region in three groups. Results shown are the representatives of three animals in each group. A: S group at 6 h; B: IR group at 6 h; C: PD group at 6 h. Scale bar, 10 μ m in A, 20 μ m in B and C.

Table 3. The expression levels of phospho-APE/Ref-1 at each time point after reperfusion in both CA1 and CA3 regions in three groups

Groups		2 h	6 h	12 h	24 h	48 h	72 h
CA1	S group	122 $\pm$ 21*	129 $\pm$ 20*	125 $\pm$ 20*	129 $\pm$ 19*	127 $\pm$ 21*	121 $\pm$ 21*
	IR group	106 $\pm$ 23*	105 $\pm$ 21*	105 $\pm$ 20*	150 $\pm$ 22*	205 $\pm$ 25	207 $\pm$ 26
	PD group	145 $\pm$ 21 ^{#Δ}	144 $\pm$ 23 ^{#Δ}	146 $\pm$ 21 ^{#Δ}	204 $\pm$ 23 ^{#Δ}	207 $\pm$ 24 ^Δ	207 $\pm$ 23 ^Δ
CA3	S group	128 $\pm$ 23*	126 $\pm$ 25*	128 $\pm$ 23*	126 $\pm$ 20*	127 $\pm$ 19*	127 $\pm$ 21*
	IR group	111 $\pm$ 25*	110 $\pm$ 24*	112 $\pm$ 21*	156 $\pm$ 22*	208 $\pm$ 23	207 $\pm$ 22
	PD group	155 $\pm$ 24 ^{#Δ}	152 $\pm$ 23 ^{#Δ}	157 $\pm$ 23 ^{#Δ}	202 $\pm$ 20 ^{#Δ}	210 $\pm$ 24 ^Δ	210 $\pm$ 25 ^Δ

* $P < 0.05$ vs PD group, [#] $P < 0.05$ vs IR group, ^Δ $P < 0.05$ vs S group, ($n = 30$, mean $\pm$ SD).

Fig. 4 Immunohistochemical images of phospho-APE/Ref-1 in CA1 region in three groups. Results shown are the representatives of three animals in each group. A: S group at 6 h; B: IR group at 6 h; C: PD group at 6 h. Scale bar, 30 μ m.

4 Discussion

In the present study, we employed mainly two assays to detect cell apoptosis: HE staining and TUNEL staining. HE staining can assess apoptosis based on cell nucleus coloring and the formation of apoptotic body, the latter of which only represents one stage during apoptosis, thus it has limitations in efficiently identifying apoptosis. TUNEL staining is a more sensitive and efficient method by identifications of different morphologic characteristics, according to previous reports^[9]. At present, CA1 region is considered to be ischemic sensitive and CA3 is ischemic tolerant. Pd98059 is a specific inhibitor of MEK protein kinase which lies in upstream of ERK signal pathway, and by inhibition of ERK phosphorylation, pd98059 may block this signal transduction pathway.

As an inducer of apoptosis, ischemia reperfusion stimulates the cell transmembrane signal transduction pathway and expressions of some genes, and acts through controlling the balance between apoptotic and anti-apoptotic genes. While previous research was mainly focused on trans membrane signal transduction pathway and repair of DNA damage, the relationship between them was seldom investigated. Our study originally connected them together and further investigated the mechanisms underlying neuron apoptosis in cerebral ischemia-reperfusion models.

Many studies indicated that APE/Ref-1 participated in neuron DNA repair and possessed anti-apoptosis roles. Gillardon *et al.*^[3] indicated that one main function of APE/Ref-1 was repairing DNA damage after cerebral ischemia. And in cerebral ischemia-reperfusion models, APE/Ref-1 played a significant role in repairing neuron basic group injury and DNA chain rupture^[4]. Being the rate-limiting enzyme during reparation of ROS injury, AP-endonuclease in APE/Ref-1 possesses the function of base excision repair, and thus repairs oxidation-induced gene damage. In addition, APE/Ref-1 participates in redox reactions through regulating the DNA binding activities of many transcription factors (AP-1, NF κ B etc), acting as a transcription factor and participating in neuron DNA repair^[5], and thus APE/Ref-1 protects neurons from apoptosis. And apoptosis is just one of the important mecha-

nisms of cerebral ischemic reperfusion injury^[11].

Phospho-ERK was present in CA3 area of ischemic tolerant region in IR group but was absent in PD group, indicating that ERK exerted cytoprotective function, which could explain the weaker expression of phospho-ERK in PD group. Meanwhile, TUNEL staining revealed that apoptotic neurons in PD group were much more than those in IR group at various time points, which further confirmed that inhibition of ERK pathway could exacerbate the cell apoptosis, as reported in other studies^[6] that ERK pathway may participate in cell protection after ischemia. Wang YQ *et al.*^[7,8] found that ischemia for 5 min could induce the up-regulation of phospho-JNK in CA1 region, with no alterations in phospho-ERK expression. And in CA3 region, phospho-ERK was detected in neurons, while phospho-JNK was detected in a much lower level. Thus it is assumed that neurons in CA1 region lack protection and the delayed neuronal death could be attributed to strengthened harmful signals. Using western blot and laser confocal microscope, Hu *et al.*^[9] found that phospho-ERK was expressed in CA3/DG region at 30 min and 4 h post ischemic reperfusion, but it was absent in CA1 neurons, indicating that ERK took part in protecting cells from ischemia injury.

In conclusion, our study revealed that APE/Ref-1 could repair DNA damage and protect neurons from apoptosis mainly in early stage after CIR (before 24 h). In addition, when the ERK pathway was inhibited, protein level of phospho-APE/Ref-1 was also decreased, and the activation of ERK signal transduction pathway could strengthen APE/Ref-1's role in repairing DNA damage. Our report indicates that ERK signal transduction pathway can protect neurons from apoptosis in cerebral ischemia and reperfusion.

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细胞信号通路与 DNA 损伤修复的关系及在大鼠全脑缺血再灌注中的作用

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摘要: 目的 观察全脑缺血再灌注时, 细胞外信号调节激酶(ERK)与无嘌呤/无嘧啶核酸内切酶/氧化还原因子-1(APE/Ref-1)蛋白在大鼠海马区神经元的表达。将跨细胞膜信号转导与胞内DNA损伤修复相联系, 探讨ERK信号通路在大鼠全脑缺血再灌注中的作用。方法 90只雄性SD大鼠随机分为3组: 假手术组(S组)、全脑缺血再灌注组(IR组)、pd98059抑制剂组(PD组)。采用四血管阻断法建立大鼠全脑缺血再灌注模型, 脑缺血时间5 min, 再灌注后2、6、12、24、48、72 h, 用免疫组化法分别检测磷酸化ERK和APE/Ref-1蛋白的表达, HE和TUNEL染色观察神经元凋亡。结果 IR组CA1区TUNEL阳性细胞于缺血再灌注6 h后开始出现, 24 h至48 h达高峰, TUNEL阳性细胞在PD组表达最强。IR组磷酸化ERK于再灌注2 h后, 在海马CA3区可见明显表达, 再灌注6 h后逐渐下降, 至再灌注后48 h未见, PD组表达弱于IR组。IR组的APE/Ref-1蛋白于再灌注后2 h在海马CA1区可见表达, 2 h到12 h表达无明显变化, 再灌注后24 h明显减少, 之后表达逐渐下降, PD组表达弱于IR组。磷酸化ERK表达下降的同时伴随有细胞凋亡的增加, APE/Ref-1在缺血再灌注后的表达变化与p-ERK一致, 并且随着ERK磷酸化的被抑制APE/Ref-1的表达更加减弱。结论 缺血再灌注中ERK通路的激活能增加APE/Ref-1蛋白的表达, 对DNA的修复起支持作用, 并在大鼠脑缺血再灌注时发挥抗神经元凋亡的保护作用。

关键词: 脑缺血再灌注损伤; ERK; APE/Ref-1; DNA修复; 凋亡