Hydroxyethylpuerarin attenuates focal cerebral ischemia-reperfusion injury in rats by decreasing TNF-α expression and NF-κB activity

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Abstract: This study is to investigate the effect of hydroxyethylpuerarin on the expression of tumor necrosis factor-alpha (TNF-α) and activity of nuclear factor kappa B (NF-κB) after middle cerebral artery occlusion (MCAO) in rats. Rats were subjected to cerebral ischemia-reperfusion injury induced by MCAO. Hydroxyethylpuerarin (10, 20, 40 mg·kg^{-1}, iv) was administered just 30 min before occlusion and immediately after reperfusion. After a 24 h reperfusion following 2 h of MCAO, the number of viable neurons in hippocampal CA1 region was counted by hematoxylin and eosin (HE) staining. TNF-α protein and its mRNA expression were examined with radioimmunoassay (RIA) and reverse transcriptase-polymerase chain reaction (RT-PCR) respectively. NF-κB activity was observed by electrophoretic mobility shift assay (EMSA), and inhibition of NF-κB α (IκBα) protein expression was evaluated by Western blotting analysis. Animals treated with hydroxyethylpuerarin had a significant increase in neuronal survival in comparison with vehicle-treated group. Hydroxyethylpuerarin significantly reduced the protein and mRNA expression of TNF-α following 2 h of ischemia with 24 h of reperfusion. NF-κB DNA binding activity and the degradation of IκBα in the cytoplasm also decreased by hydroxyethylpuerarin treatment. The protective effects of hydroxyethylpuerarin against ischemia-reperfusion injury may be mediated by decreasing the expression of TNF-α and the activity of NF-κB in rats.

Key words: hydroxyethylpuerarin; cerebral ischemia; tumor necrosis factor-alpha; nuclear factor kappa B

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羟乙葛根素对大鼠脑缺血再灌注损伤后 TNF-α表达及 NF-κB活性的影响

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摘要: 观察羟乙葛根素对大鼠局灶性脑缺血再灌注损伤后 TNF-α表达及 NF-κB活性的影响。采用大鼠大脑中动脉内栓线阻断法(MCAO)建立大鼠脑缺血再灌注损伤模型,分别于缺血前 30 min及再灌注即刻由尾静脉注射羟乙葛根素(10, 20及 40 mg·kg^{-1}),缺血 2 h再灌注 24 h后取缺血侧脑组织, HE染色观察大鼠脑组织病理学变化并计数海马 CA1区存活神经元数目,放射免疫分析测定脑组织匀浆中 TNF-α含量,逆转录聚合酶链式反应(RT-PCR)测定脑组织中 TNF-α mRNA表达情况,凝胶电泳迁移率实验(EMSA)观察 NF-κB DNA结合活性改变, Western blotting检测观察 IκBα蛋白表达情况。羟乙葛根素可明显改善大鼠海马 CA1区损伤程度,升高锥体存活神经元数目,减少 TNF-α蛋白及 mRNA表达,抑制 NF-κB DNA结合活性。羟乙葛根素可减轻大鼠脑缺血再灌注损伤后炎症反应,这可能是其发挥脑保护作用的机制之一。

关键词: 羟乙葛根素; 脑缺血; 肿瘤坏死因子-α; 核因子-κB

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Many studies have shown that the inflammatory reactions play a key role in the second injury after acute cerebral ischemia. It is characterized by infiltration of leukocytes in the brain tissue. Proinflammatory cytokines such as TNF-α and IL-1β, the important signal transduction molecules in the process of inflammatory response, are upregulated after ischemia-reperfusion injury. During the process of ischemia-reperfusion, TNF-α can attract leukocytes, stimulate the expression of adhesion molecules in leukocytes, endothelial cells and other cells and promote the inflammatory response of ischemic brain tissue[1]. NF-κB, a proinflammatory transcription factor and closely associated with ischemia-reperfusion injury, is activated in the ischemic brain tissue[2].

Recent study has revealed that puerarin could exhibit its neuroprotective effect by inhibiting the inflammatory reactions in the brain tissue[3]. But the lipid solubility of puerarin is comparatively low, which limits its effectiveness to some extent. Hydroxyethylpuerarin, synthesized by structural modification of puerarin, has higher lipid solubility and blood brain barrier (BBB) permeability as compared to puerarin[4]. Our previous studies reported that hydroxyethylpuerarin could protect brain against ischemia-reperfusion injury and reduce infarct size by decreasing the damage of oxygen free radicals and increasing the activity of antioxidase[5]. It could also protect cerebral microvascular endothelial cells and astrocytes against the injury induced by hydrogen peroxide (H_2O_2) *in vitro*[4,6]. However, the precise mechanism of action of hydroxyethylpuerarin is still not clear.

In this study, the effects of hydroxyethylpuerarin on TNF-α expression and NF-κB activity in the ischemic brain tissue were observed to explore the potential neuroprotective mechanism of hydroxyethylpuerarin.

Materials and methods

Drugs and reagents Hydroxyethylpuerarin was supplied by Shandong Academy of Medical Sciences. Rabbit polyclonal anti-IκBα antibody, goat polyclonal anti-actin antibody, peroxidase conjugated goat anti-rabbit IgG, rabbit anti-goat IgG and Western blotting luminol reagent were purchased from Santa Cruz Biotechnology. RNA PCR kit was purchased from Takara. Light shift™ chemiluminescent EMSA kit was purchased from Pierce (No. 20148).

Induction of ischemia and drug treatment Adult male Wistar rats (Grade II, certificate No. 2001003, purchased from the Laboratory Animal Center, Shandong University) weighing 270 - 320 g were used for experiment. Middle cerebral artery (MCA) occlusion was prepared as previously described[7]. Briefly, rats were anesthetized with 10% chloral hydrate (350 mg• kg^{-1}, ip). The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. An 18 mm length of nylon suture (φ 0.2 mm) was introduced into the ECA lumen and advanced into the ICA to block the origin of the MCA. Restoration of MCA blood flow in animals subjected to 2 h of MCA occlusion was achieved by withdrawing the suture to the ECA. The sham control rats received the same surgery procedures but did not have the suture inserted.

Hydroxyethylpuerarin (10, 20, 40 mg• kg^{-1}, iv) was administered just 30 min before occlusion and immediately after reperfusion. Control rats were received vehicle (0.9% NaCl, iv). Rats were sacrificed and the ischemic brain tissues were immediately removed 24 h after reperfusion. Part samples were isolated for routine pathological examination and counting the number of viable neurons in hippocampal CA1 region, the rest were immediately frozen in liquid nitrogen for future use.

Histological analysis After ischemia-reperfusion (I/R) injury or sham-operation, the rats were anesthetized with 10% chloral hydrate (350 mg• kg^{-1}, ip), then the rats were transcardially perfused with 100 mL of 0.9% NaCl solution and subsequently with 4% paraformaldehyde in 0.1 mol• L^{-1} phosphate buffer at pH 7.4. Brains were removed and post-fixed for 24 h in 10% formalin. Paraffin sections were prepared and stained with hematoxylin and eosin. The sections were examined with a light microscope and the number of the surviving hippocampal CA1 neurons was counted.

Measurement of TNF-α content Brain tissue samples (100 mg) obtained from the ischemic cortex were used to measure TNF-α content. The procedure used to quantify TNF-α content from rat brain samples was according to the guidelines provided with the RIA kit (Beijing North Institute of Biological Technology, China).

RT-PCR analysis Total RNA were extracted from the brain tissues using the Trizol extraction kit.

RNA concentration was determined by spectrophotometry at 260 nm. RT-PCR was carried out with the RNA PCR Kit. The reverse transcription mixture (20 μL) contained total RNA 1 μg, 10 × RNA PCR buffer 2 μL, 5 mmol· L^{-1} $MgCl_2$, 1 mmol· L^{-1} dNTP, Oligo dT 2.5 pmol, AMV 5 U and RNase inhibitor 20 U. After mixing, the system was incubated at 30 ℃ for 10 min, 42 ℃ for 30 min, 99 ℃ for 5 min and 5 ℃ for 5 min. PCR was performed to assess the expression of TNF-α mRNA using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The oligonucleotide primers specific for rat TNF-α (forward: 5′-CTC TTC AAG GGA CAA GGC TG-3′, reverse: 5′-TCA CAG AGC AAT GAC TCC AAA G-3′, 285 bp) and GAPDH (forward: 5′-TCC CTC AAG ATT GTC AGC AA-3′, reverse: 5′-AGA TCC ACA ACG GAT ACA TT-3′, 307 bp) were synthesized from Sangon. PCR conditions were as follows: 1 μg of cDNA mixture was subjected to amplification in 50 μL of final volume with 10 × PCR buffer 2 μL, 1.75 mmol· L^{-1} $MgCl_2$, 200 μmol· L^{-1} dNTP, Taq DNA polymerase 1.25 U and 50 pmol of each primer in the reaction buffer. PCR cycles were as follows: an initial hot start (4 min at 95 ℃) followed by denaturation at 94 ℃ for 30 s, annealing at 60.5 ℃ for 30 s, extension at 72 ℃ for 30 s for 35 cycles, and a 5 min final extension period at 72 ℃. The PCR products were subjected to 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. The density of each band was measured by a densitometer. The semiquantitative measure was expressed as ratios compared with GAPDH.

Preparation of cytoplasmic and nuclear extracts The cytoplasmic and nuclear protein extracts were prepared according to the protocols of Huang et al[8] with some modifications. Briefly, tissue samples were homogenized in ice-cold buffer A [150 mmol· L^{-1} NaCl, 10 mmol· L^{-1} HEPES-KOH (pH 7.9), 0.5 mmol· L^{-1} phenylmethylsulfonyl fluoride (PMSF), 1 mmol· L^{-1} edetic acid (EDTA, pH 8.0) and 0.6% NP-40]. After a 5 min incubation on ice, the homogenates were centrifuged at 10 000 × *g* for 10 min at 4 ℃. The supernatant fluid (cytoplasmic extracts) was collected and stored at - 80 ℃ for Western blotting analysis of IκBα protein expression. The nuclear pellet was resuspended in buffer B [420 mmol· L^{-1} NaCl, 20 mmol· L^{-1} HEPES-KOH (pH 7.9), 0.5 mmol· L^{-1} PMSF, 0.2 mmol· L^{-1} EDTA (pH 8.0), 0.5 mmol· L^{-1} dithiothreitol (DTT), 1.2 mmol· L^{-1} $MgCl_2$, 25% glycerol and 0.5 mg· L^{-1} aprotinin] and was left for 30 min on ice with constant agitation. After centrifugation for 15 min at 4 ℃, the nuclear extracts (supernatants) used for electrophoretic mobility shift assay (EMSA) of NF-κB activity were stored in aliquots at - 80 ℃ until used for analysis. The protein concentration was determined by Bradford protein assay with bovine serum albumin as the standard.

Electrophoretic mobility shift assay (EMSA) EMSA was carried out with the Light shift™ chemiluminescent EMSA kit. The specific NF-κB DNA probes (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′) were 5′-end labeled with biotin and annealed to double strand. The 20 μL binding reaction system including 10 × binding buffer 2 μL, 50% glycerol 1 μL, 100 mmol· L^{-1} $MgCl_2$ 1 μL, 1 g· L^{-1} poly(dI: dC) 1 μL, 1% NP-40 1 μL, nuclear extracts 10 μg, biotin end-labeled NF-κB DNA probes 20 fmol were incubated at room temperature for 20 min, then mixed with 5 × loading buffer 5 μL and electrophoresed on 5% polyacrylamide gel with 0.5 × TBE, and finally were electroblotted onto positively-charged nylon membranes (Amersham). After the transferred DNA were cross-linked to the nylon membrane, the biotin-labeled DNA were detected using the protocol in the kit, which was as follows: the membrane was incubated in the Light shift™ blocking buffer and the conjugate/blocking buffer for 15 min successively, after washed for 4 times, the membrane was incubated in the Light shift™ substrate equilibration buffer for 5 min, then in the Light shift™ substrate working solution for 5 min, finally the membrane was placed in a film cassette and exposed to X-ray film for 2 - 5 min.

The specificity of NF-κB binding was confirmed by adding 200-fold excess of unlabeled NF-κB DNA probe to the assay.

Western blotting analysis Samples were mixed with loading buffer and boiled for 5 min, cytoplasmic extracts (60 μg total protein/lane) were separated by 10% SDS-PAGE followed by electroblotting onto nitrocellulose (NC) membranes (Amersham). After blocked overnight at 4 ℃ in TBS with 0.1% Tween-20 (TBS-T) and 5% non-fat dried milk, membranes were incubated overnight with primary antibody (anti-IκBα antibody 1∶100; anti-actin antibody 1∶100) in blocking buffer at 4 ℃. The membrane were then

washed five times with TBS-T and incubated with secondary peroxidase-conjugated antibody (1∶1 500) in blocking buffer. After successive washes, the membranes were detected with an enhanced chemiluminescence kit. The density of each band was measured by a densitometer, the semiquantitative measure was expressed as ratio compared with actin.

Statistics RT-PCR, EMSA, and Western blotting results were semiquantitatively evaluated by means of an imager analyzer (AlphaImager 2002). Data were expressed as $\bar{x} \pm s$ and statistical analysis of the results was carried out by one-way analysis of variance (ANOVA). The level of the statistical significance was set at $P < 0.05$.

Results

1 Protective effect of hydroxyethylpuerarin against I/R injury

As shown in Table 1, the number of survival neurons in hippocampus CA1 region decreased significantly in I/R group in comparison with that in sham-operated group ($P < 0.01$), while animals treated with hydroxyethylpuerarin 40 and 20 mg• kg^{-1} had a significant increase in viable neurons in comparison with vehicle-treated rats ($P < 0.01$).

Table 1 Effect of hydroxyethylpuerarin on the number of survival hippocampal CA1 neurons after 24 h reperfusion following 2 h of MCAO in rats

Group	Dose /mg• kg^{-1}	Survival neuron
Sham		221 ±22
I/R		94 ±17**
Hydroxyethylpuerarin	40	172 ±42$^{\#\#}$
	20	156 ±20$^{\#\#}$
	10	111 ±24
Puerarin	18	129 ±17

$n = 6$, $\bar{x} \pm s$. ** $P < 0.01$ *vs* sham group; $^{\#\#}$ $P < 0.01$ *vs* I/R group

2 Effect of hydroxyethylpuerarin on TNF-α content in ischemic brain tissue

After a 24 h reperfusion following 2 h of MCAO, TNF-α content increased greatly in the vehicle-treated group in comparison with that in sham-operated group ($P < 0.01$). Hydroxyethylpuerarin treatment significantly suppressed the increase of TNF-α content induced by MCAO ($P < 0.05$ or $P < 0.01$, Table 2).

3 Effect of hydroxyethylpuerarin on TNF-α mRNA expression in ischemic brain tissue

Semiquantitative RT-PCR with GAPDH as an internal control was used to characterize the TNF-α mRNA in the infarct cortex (Figure 1). The mRNA expression of TNF-α increased significantly in I/R group. The expression was significantly reduced by hydroxyethylpuerarin 40, 20 and 10 mg• kg^{-1} ($P < 0.01$, Table 2).

Table 2 Effect of hydroxyethylpuerarin on TNF-α content and mRNA expression in the brain tissue after 24 h reperfusion following 2 h of MCAO in rats

Group	Dose /mg• kg^{-1}	TNF-α content /nmol• L^{-1}	TNF-α mRNA expression
Sham		2.57 ±0.53	0.25 ±0.05
I/R		14.82 ±1.58**	0.86 ±0.07**
Hydroxyethylpuerarin	40	6.91 ±1.03$^{\#\#}$	0.31 ±0.02$^{\#\#}$
	20	10.32 ±1.14$^{\#\#}$	0.43 ±0.05$^{\#\#}$
	10	12.86 ±1.16$^{\#}$	0.50 ±0.09$^{\#\#}$
Puerarin	18	9.02 ±0.79$^{\#\#}$	0.43 ±0.06$^{\#\#}$

$n = 6$, $\bar{x} \pm s$. ** $P < 0.01$ *vs* sham group; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ *vs* I/R group

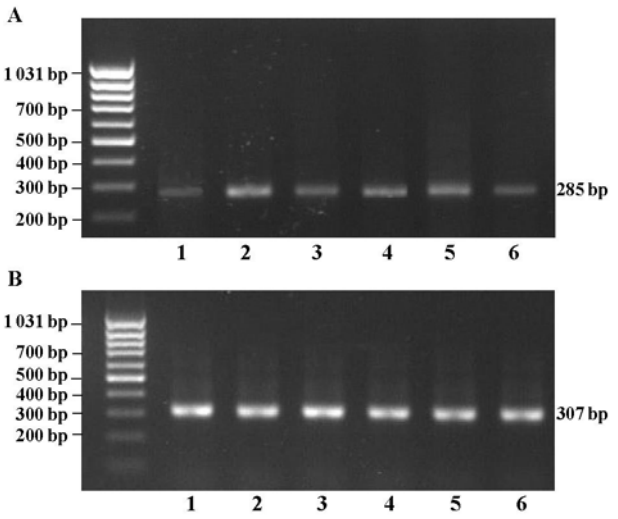

Figure 1 RT-PCR analysis of TNF-α (285 bp, A) and GAPDH (307 bp, B) mRNA in cerebral cortex in MCAO rats. Lane 1: Sham group; Lane 2: I/R group; Lane 3 - 5: Hydroxyethylpuerarin 40, 20, 10 mg• kg^{-1} group; Lane 6: Puerarin 18 mg• kg^{-1} group

4 Effect of hydroxyethylpuerarin on NF-κB activity

As shown in Figure 2 and Table 3, the ischemia-induced increase of NF-κB DNA binding activity in ischemic cortex of nuclear extracts was significantly inhibited by treatment with hydroxyethylpuerarin 20 and 40 mg• kg^{-1} ($P < 0.05$ or $P < 0.01$).

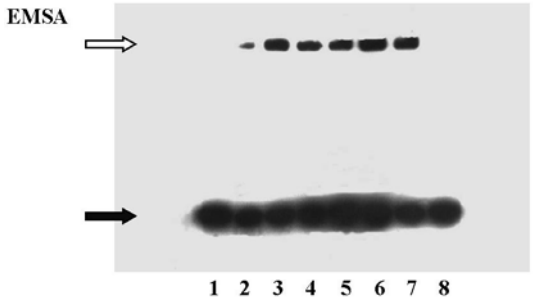

Figure 2 Effect of hydroxyethylpuerarin on NF-κB activity by EMSA analysis in the brain ischemic area in MCAO rats. Lane 1: Free probe; Lane 2: Sham group; Lane 3: I/R group; Lane 4 - 6: Hydroxyethylpuerarin 40, 20, 10 mg·kg^{-1} group; Lane 7: Puerarin 18 mg·kg^{-1} group; Lane 8: Hydroxyethylpuerarin 40 mg·kg^{-1} group + 200-fold unlabeled NF-κB probe. The open arrowheads indicate the position of NF-κB DNA binding complexes. The filled arrowheads indicate the position of unbound DNA probes

Table 3 Effect of hydroxyethylpuerarin on I/R induced alteration of NF-κB DNA binding activity and IκBα protein expression after 24 h reperfusion following 2 h of MCAO in rats

Group	Dose /mg·kg^{-1}	NF-κB activation	IκBα /actin
Sham		0.21 ±0.04	0.45 ±0.04
I/R		1.00 ±0.00**	0.24 ±0.04**
Hydroxyethylpuerarin	40	0.52 ±0.06##	0.38 ±0.05##
	20	0.73 ±0.08#	0.35 ±0.05##
	10	0.78 ±0.09	0.30 ±0.04#
Puerarin	18	0.73 ±0.07#	0.34 ±0.04##

$n = 3$, $\bar{x} \pm s$. ** $P < 0.01$ *vs* sham group; # $P < 0.05$, ## $P < 0.01$ *vs* I/R group

5 Effect of hydroxyethylpuerarin on IκBα protein expression

IκBα protein reduced dramatically in ischemic cortex after transient MCAO in the model control group as indicated by Western blotting, whereas no significant reduction of IκBα in the sham control group was observed. The degradation of IκBα protein reduced markedly in the groups treated with hydroxyethylpuerarin ($P < 0.05$ or $P < 0.01$, Figure 3 and Table 3).

Disscusion

Proinflammatory cytokines such as IL-1 and TNF-α possess a wide range of biological activities in various tissues. In recent years, there has been increasing

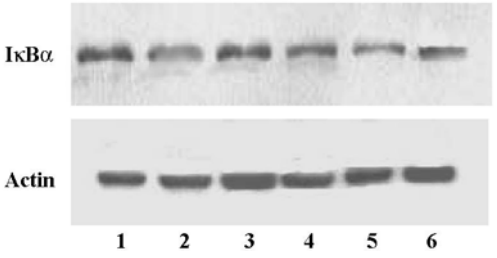

Figure 3 Effect of hydroxyethylpuerarin on the brain ischemic area in MCAO rats by Western blotting analysis with anti-IκBα antibody. Lane 1: Sham group; Lane 2: I/R group; Lane 3 - 5: Hydroxyethylpuerarin 40, 20, 10 mg·kg^{-1} group; Lane 6: Puerarin 18 mg·kg^{-1} group

evidence that these cytokines are involved in inflammatory reactions in central nervous system disease. TNF-α is one of the multi-functional proinflammatory cytokines which can be synthesized and secreted by macroglia, astrocyte and endothelial cells after brain ischemia-reperfusion injury[9]. It can stimulate the expression of adhesion molecules (including ICAM-1, VCAM-1 and E-selectin) and promote the release of IL-1 and nitric oxide that contributes to increased vascular permeability. In addition, TNF-α can stimulate the production of reactive oxygen species in endothelium by inducing the enzymes such as xanthine oxidase, cyclooxygenase (COX) and phospholipase A_2 (PLA_2). All of these effects contribute to brain ischemia-reperfusion injury[10]. Barone et al[11] showed that administration of TNF-α exacerbated the ischemic injury provoked by MCA occlusion in spontaneously hypertensive rats, and also demonstrated that anti-TNF-α antibodies had a neuroprotective effect. Similarly, the inhibition of TNF-α in mice with permanent MCA occlusion leads to a smaller infarct volume[12]. In our present study, hydroxyethylpuerarin significantly reduced the TNF-α content and mRNA expression in the ischemic region, which indicated that hydroxyethylpuerarin can exhibit its protective effects through down regulation of TNF-α expression in ischemic cortex.

Many studies demonstrated that NF-κB is continuously activated following focal brain ischemia injury. NF-κB is an oxidative responsive transcription factor that can be activated by ROS, cytokines or virus. When activated, it can induce a number of target genes including those encoding cytokines, cell adhesion molecules and acute phase proteins. NF-κB usually locates in the cytoplasm as an inactive

multisubunit complex associated with an inhibitory subunit IκB (the usual form is IκBα). When cell is stimulated, NF-κB is activated and translocates into the nucleus by phosphorylation and degradation of IκB, where it binds to a κB-specific DNA motif and regulates transcription of target genes[13]. Therefore, modulation of NF-κB activation may provide a direct way of inhibiting inflammatory mediators. In our study, both the ischemic-induced NF-κB activation in the nucleus and the IκBα degradation in the cytoplasm can be blocked by hydroxyethylpuerarin treatment, which suggests that hydroxyethylpuerarin may exhibit its protective effects against ischemia-reperfusion injury by down-regulation of NF-κB activation.

In conclusion, hydroxyethylpuerarin can decrease the protein and mRNA expression of TNF-α, inhibit the NF-κB DNA binding activity in the nucleus and prevent the loss of IκBα in the cytoplasm. The protective effects of hydroxyethylpuerarin against ischemia-reperfusion injury may be mediated by down-regulation of NF-κB activation.

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