Anti-inflammatory Effect of Hydroxyethylpuerarin on Focal Brain Ischemia/Reperfusion Injury in Rats

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Abstract

The objective of this study is to investigate the anti-inflammatory effect of hydroxyethylpuerarin on focal brain ischemia injury in rats and to explore its mechanisms of action. After 24 h of reperfusion following 2 h of cerebral ischemia, the infiltration of neutrophils was observed by myeloperoxidase (MPO) activity determination, the expression of intercellular adhesion molecule-1(ICAM-1) was observed by western blot and reverse transcriptase-polymerase chain reaction(RT-PCR) analysis, and the nuclear translocation and DNA binding activity of nuclear factor-κB (NF-κB)were observed by western blot and electrophoretic mobility shift assay (EMSA). The results showed that hydroxyethylpuerarin could obviously inhibit the MPO activity and ICAM-1 expression following 2 hours of ischemia with 24 hours of reperfusion. The nuclear translocation and DNA binding activity were also decreased by hydroxyethylpuerarin treatment. These results suggested that hydroxyethylpuerarin could inhibit neutrophil-mediated inflammatory response after brain ischemia reperfusion in rats. This effect may be mediated by down-regulation of ICAM-1 and NF-κB activity.

Key Words: hydroxyethylpuerarin; myeloperoxidase; intercellular adhesion molecule-1; nuclear factor -κ B; brain ischemia injury

Introduction

There is now considerable evidence indicating that inflammatory mechanisms play an important role in the secondary injury after acute cerebral ischemia, which was characterized by a progressive increase in neutrophils adhesion and infiltration. Neutrophils contribute to the secondary injury by releasing reactive oxygen species (ROS), generating lipid mediators, disrupting the blood-brain barrier(BBB), increasing cerebral edema and plugging the cerebral microvasculature. Recent studies have shown that blocking neutrophils infiltration could reduce ischemia injury and that the expression of ICAM-1, which is mainly involved in neutrophils adhesion and migration, is also up-regulated in ischemic brain tissue (10).

Hydroxyethylpuerarin, which was modified on the structural basis of puerarin, has higher lipid solubility and BBB permeability than puerarin. Our previous study has shown that hydroxyethylpuerarin could exhibit its protective effects against brain ischemia-reperfusion injury by reducing infarct size, reducing damage of oxygen free radicals, increasing the activity of antioxidase, and inhibiting cell apoptosis in ischemic area (8, 9). However, further information of mechanisms of hydroxyethylpuerarin still awaits discovery.

In the present study, we observed the effects of hydroxyethylpuerarin on MPO activity, the expression of ICAM-1 and NF-κB activity in the ischemic brain tissue to study the potential neuroprotetive mechanism of hydroxyethylpuerarin.

Materials and Methods

Drugs and Reagents

Hydroxyethylpuerarin was provided by Shandong Academy of Medical Sciences, LOT:

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010407. Mouse monoclonal anti-ICAM-1, anti-p65 antibodies, goat polyclonal anti-actin antibody, peroxidase conjugated goat anti-mouse IgG, rabbit anti-goat IgG and western blot luminol reagent were purchased from Santa Cruz Biotechnology. RNA PCR Kit were purchased from Takara. Light shift™ chemiluminescent EMSA kit were purchased from PIERCE. MPO activity determination kit was purchased from Nanjing Jiancheng Bioengineering Institute. Nitrocellulose membrane and positively charged nylon membrane were purchased from Amersham Biosciences.

Animal Preparation and Drug Treatment

Adult male Wistar rats (clean grade, certificate No. 2001003, purchased from Laboratory Animal Center, Shandong University) weighing 270~320g were used for experiment. Middle cerebral artery (MCA) occlusion was induced as previously described (5). Briefly, rats were anesthetized with 10% chloralhydrate (350 mg/kg, 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, iv) was administered just 30 min before occlusion and immediately after reperfusion. Control rats received vehicle (0.9% NaCl, iv). Twenty-four hours after reperfusion, rats were sacrified and the ischemic brain tissues were immediately frozen in liquid nitrogen for late use.

MPO Activity

MPO activity was used as an indicator of tissue neutrophils accumulation following cerebral ischemia (6). Tissue samples (200 mg) obtained from the center of the MCA distribution were used to measure the MPO activity. The procedures used to quantify MPO activity from rat brain samples were performed according to the description of the kit.

mRNA Expression of ICAM-1: 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, $MgCl_2$ 5 mmol/L, dNTP 1 mmol/l, Oligo dT 2.5 pmol, AMV 5 U, RNase inhibitor 20 U. After mixing, the system was incubated at 30˚C for 10 min, 42˚C for 30 min, 99˚C for 5 min, and 5˚C for 5 min. PCR was performed to assess the expression of ICAM-1 mRNA using GAPDH as an internal control. The oligonucleotide primers specific for rat ICAM-1 (forward: 5'-GGC GTC CAT TTA CAC CTA TTA-3', revese: 5'-TTC CTT TTC TTC TCT TGC TTG-3'; 413bp) and GAPDH (forward: 5'-TCC CTC AAG ATT GTC AGC AA-3', revese: 5'-AGA TCC ACA ACG GAT ACA TT-3'; 307bp) were synthesized from Sangon. PCR conditions were as follows: 2.5 μl of cDNA mixture was subjected to amplification in 50 μl of final volume with 10×PCR buffer 2 μl, $MgCl_2$ 2 mmol/l, dNTP 200 μmol/l, 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˚C) followed by denaturation at 94˚C for 30 sec, annealing at 57˚C for 45 sec, extension at 72˚C for 45 seconds for 35 cycles, and 5 min of final extension period at 72˚C. 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. This 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 (4) with some modifications. Briefly, tissue samples were homogenized in ice-cold buffer A (NaCl 150 mmol/l, HEPES-KOH, pH 7.9, 10 mmol/l, phenylmethylsulfonyl fluoride (PMSF) 0.5 mmol/l, edetic acid (EDTA), pH 8.0, 1 mmol/l, NP-40 0.6%). After 5 min incubation on ice, the homogenates were centrifuged (5000 rpm, 10 min) at 4˚C. The supernatant fluid (cytoplasmic extracts) was collected and stored at -80˚C for western blot analysis of ICAM-1 protein expression. The nuclear pellets were resuspended in buffer B (NaCl 420 mmol/l, HEPES-KOH, pH 7.9, 20 mmol/l, PMSF 0.5 mmol/l, EDTA, pH 8.0 0.2 mmol/l, dithiothreitol (DTT) 0.5 mmol/l, $MgCl_2$ 1.2 mmol/l, glycerol 25%, aprotinin 0.5 μg/ml) and were left for 30 min on ice with constant agitation. After centrifugation for 15 min, 14000 rpm at 4˚C, nuclear extracts were stored in aliquots at -80˚C. Nuclear extracts were used for western blot analysis of p65 protein expression and for electrophoretic mobility shift assay (EMSA) of NF-κB activity. The protein concentration was determined by Bradford protein assay kit.

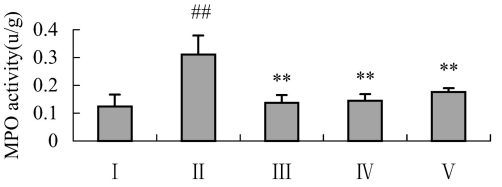

Fig. 1. Effects of hydroxyethylpuerarin on myeloperoxidase activity in the brain ischemic area after 24 h reperfusion following 2 h MCA occlusion in rats (mean±s, n= 8).**P < 0.01 *vs*. model control group (I/R), ##P < 0.01 *vs*. sham group. Lane I sham Lane II I/R Lane III, IV, V: hydroxyethylpuerarin 40, 20, 10 mg/kg.

Western Blot Analysis for ICAM-1 and p65

Samples were mixed with loading buffer and boiled for 5 min, cytoplasmic extracts (150 μg total protein/lane) and nuclear extracts (30 μg total protein/ lane) were separated by 7.5% and 10% SDS-PAGE gel electrophoresis separately and were then electroblotted onto nitrocellose (NC) membranes. The membranes were blocked (2 h, 4˚C) in 5% non-fat dried milk in TBS-T, then incubated with primary antibody (anti-ICAM-1 antibody 1:100; anti-actin antibody 1:100; anti-p65 antibody 1:100) in blocking buffer overnight at 4˚C and washed five times with TBS-T before incubation with secondary peroxidase-conjugated antibody (1:1000) in blocking buffer. After successive washes, the membranes were detected with an enhanced chemilumiescence kit.

DNA Binding Activity of NF-κB: Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out with the Light shift™ Chemiluminescent EMSA kit (PIERCE, NO.20148). The specific NF-κB DNA probes 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 mM $MgCl_2$ 1 μl, 1 μg/μl poly (dI:dC) 1 μl, 1% NP-40 1 μl, 10 μg nuclear extracts, and 20 fmol biotin end-labeled NF-κB DNA probes were incubated at room temperature for 20 min, and then mixed with 5 μl 5×loading buffer and electrophoresed on 5% polyacrylamide gel with 0.5×TBE. Next, they were electroblotted onto positively-charged nylon membranes. After the transferred DNA were cross-linked to the nylon membrane, the biotin-labelled DNA were detected according to the kit.

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

Statistics

Data were expressed as mean±SD and compared with ANOVA, followed by Student's *t*-test. The level of the statistical significance was set at P < 0.05.

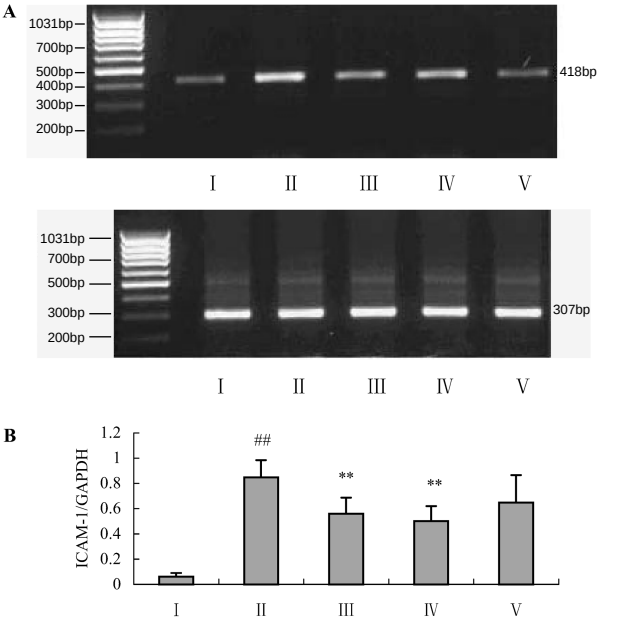

Fig. 2. A. RT-PCR analysis of ICAM-1 (413 bp) and GAPDH (307 bp) mRNA in cerebral cortex in MCAO rats.
B. Effects of hydroxyethylpuerarin on expression of ICAM-1 mRNA in the brain ischemic area in MCAO rats. n=6, ##P < 0.01 *vs*. sham group, **P < 0.01 *vs*. model control group (I/R)
Lane I: sham lane II: I/R lane III, IV, V: hydroxyethylpuerarin 40, 20, 10 mg/kg.

Results

MPO Activity

After 24 h reperfusion following 2 h MCA occlusion, MPO activity was greatly increased in ischemia/reperfusion group rats. Hydroxyethylpuerarin treatment significantly suppressed the MPO activity induced by MCAO (Fig. 1)

ICAM-1 mRNA Expression: RT-PCR Analysis

Semiquantative RT-PCR with GAPDH as an internal control was used to characterize the ICAM-1 mRNA in the infarct cortex (Fig. 2A). The mRNA expression of ICAM-1 increased greatly in I/R group. The expression was significantly reduced by hydroxyethylpuerarin 40 and 20 mg/kg. (Fig. 2B).

Western Blot Analysis for ICAM-1 and NF-κB p65 Protein Expression

ICAM-1 protein was dramatically induced in

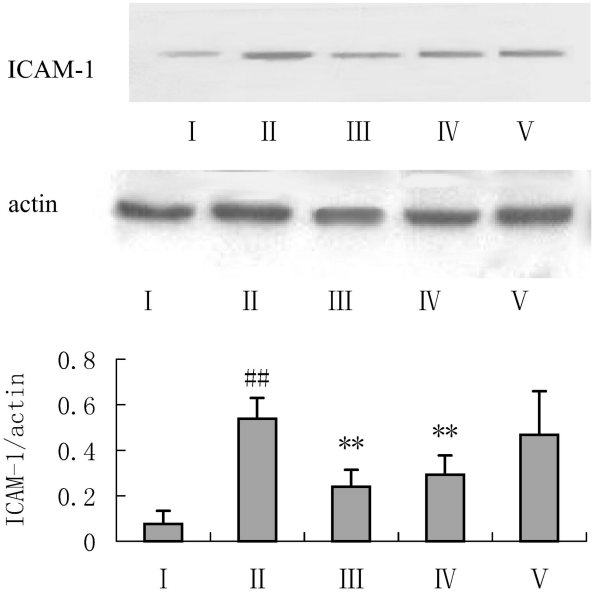

Fig. 3. Effects of hydroxyethylpuerarin on expression of ICAM-1 protein in the brain Ischemic area in MCAO rats, n=3. ##P < 0.01 *vs*. sham group, **P < 0.01 vs model control group (I/R)
Lane I: sham lane II: I/R lane III, IV, V: hydroxyethylpuerarin 40, 20, 10 mg/kg.

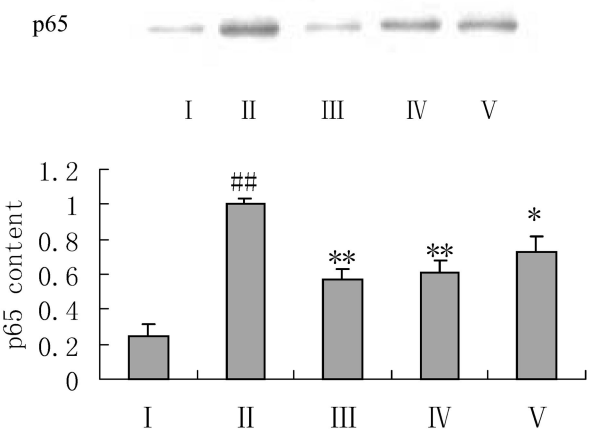

Fig. 4. Western blot analysis with anti-p65 antibody and the effects of hydroxyethylpuerarin on it, n=3. ##P < 0.01 *vs*. sham group, *P < 0.05,**P < 0.01 *vs* model control group (I/R)
Lane I: sham lane II: I/R lane III, IV, V: hydroxyethylpuerarin 40, 20, 10 mg/kg.

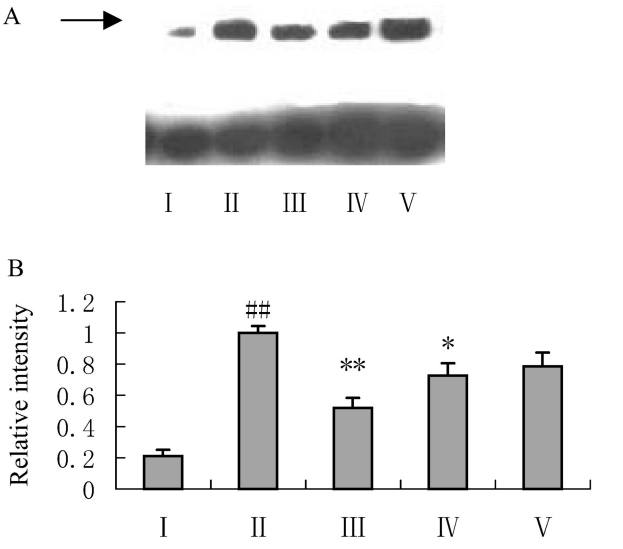

Fig. 5. A: EMSA of NF-κB DNA binding activity in rats subjected to I(2h)/R(24h) (n=3)
B: Semi-quanitative analysis of EMSA using densitometry of the band(arrow) in each lane. ##P < 0.01 vs sham group, *P < 0.05,**P < 0.01 *vs*. model control group (I/R)
Lane I: sham lane II: I/R lane III, IV, V: hydroxyethylpuerarin 40, 20, 10 mg/kg.

ischemic cortex after transient MCAO in the model control group as indicated by western blot, whereas no significant expression of ICAM-1 in the sham control group was observed. The increase in ICAM-1 protein content was markedly reduced in hydroxyethylpuerarin 40 and 20 mg/kg treated group, respectively (Fig. 3).

Western blot analysis of homogenates from nuclear extracts showed that p65 subunit was evidently increased after I (2 h)/R (24 h) following MCAO in the model control group. This nuclear translocation of NF-κB/p65 was evidently inhibited by hydroxyethylpuerarin 40, 20 and 10 mg/kg (Fig. 4).

DNA Binding Activity of NF-κB: EMSA

As shown in Fig. 5, 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.

Discussion

Post-ischemic cerebral inflammation has been implicated as a secondary injury mechanism following ischemia and stroke. The recruitment of neutrophils to the area of ischemia was the first step to inflammation. The roles of neutrophils in the pathogenesis of cerebral ischemia and stroke include reducing microvascular blood flow, initiating thrombosis, lipid mediator synthesis and active oxygen species production. Neutrophils infiltration in the area of cerebral infarction is a well-documented histopathological finding and MPO activity determination is widely used to assess the neutrophils infiltration in the laboratory studies. Our present study revealed that hydroxyethylpuerarin treatment

significantly attenuated MPO activity and the number of neutrophils infiltration in the ischemic region. This finding indicated that hydroxyethylpuerarin could reduce cerebral ischemic damage by inhibiting the post-ischemic inflammatory process.

Neutrophils adhesion to endothelium is the first step to acute inflammation during an ischemia-reperfusion injury. The adhesion of neutrophils to the endothelial surface and their subsequent migration from the microvessels into the brain parenchyma is mediated by a variety of adhesion molecules located on the surface of both neutrophils and endothelial cells. Among adhesion molecule receptors found on neutrophils, the CD11/CD18 (β_2 integrins) glycoprotein complex appeared to play a determinant role in the neutrophil-endothelium interaction. ICAM-1 is an endothelial ligand for the β_2 integrins on neutrophils. ICAM-1 is constitutively expressed at low levels on vascular endothelium and can be markedly upregulated by ischemia-reperfusion in brain microvessels. Factors that antagonize adhesion molecules have been shown to reduce reperfusion injury. For instance, anti-ICAM-1 monoclonal antibody reduced neutrophil infiltration and brain damage after reperfusion (11). there is also report that mice belonging to an ICAM-1 deficient strain show a marked reduction in cerebral infarction size after transient MCA occlusion in comparison with normal controls (3,7). In our study, hydroxyethylpuerarin was found to reduce the expression of ICAM-1, which suggest that hydroxyethylpuerarin can suppress neutrophil infiltration following ischemic insult by reducing adhesion molecules expression.

NF-κB is a proinflammatory transcription factor that is closely associated with ischemia-reperfusion injury. It is made up of two subunits. The usual form of NF-κB is heterodimer of p65 and p50. p65 contain a transactivation domain. The heterodimer normally exists in the cytoplasm in an inactive form bound to an inhibitory protein, I κB. In response to a variety of proinflammatory signals, I κB is rapidly degraded and NF-κB is activated and then translocated into the nucleus, then NF-κB binds to a κB-specific DNA motif and regulates transcription of target genes such as adhesion molecules, proinflammatroy cytokines and inflammatory enzymes (1,2). Therefore, modulation of NF-κB activation may provide a direct way of inhibiting inflammatory mediators. Our study showed that NF-κB was activated in ischemic cortex following brain ischemia and hydroxyethylpuerarin could inhibit the nuclear translocation of NF-κB as well as its DNA binding activity, which indicated hydroxyethylpuerarin may exhibit its protective effects against ischemia-reperfusion injury by down-regulation of NF-κB activation.

In conclusion, hydroxyethylpuerarin is effective in the down-regulation of MPO activity and ICAM-1 expression following focal ischemic-reperfusion injury, which may be mediated by inhibition of NF-κB activation.

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