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Effects of β-Aescin on the expression of nuclear factor-κB and tumor necrosis factor-α after traumatic brain injury in rats*

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Abstract: To investigate the inhibiting effect of β-Aescin on nuclear factor-κB (NF-κB) activation and the expression of tumor necrosis factor-α (TNF-α) protein after traumatic brain injury (TBI) in the rat brain, 62 SD rats were subjected to lateral cortical impact injury caused by a free-falling object and divided randomly into four groups: (1) sham operated (Group A); (2) injured (Group B); (3) β-Aescin treatment (Group C); (4) pyrrolidine dithocarbamate (PDTC) treatment (Group D). β-Aescin was administered in Group C and PDTC treated in Group D immediately after injury. A series of brain samples were obtained directly 6 h, 24 h and 3 d respectively after trauma in four groups. NF-κB activation was examined by Electrophoretic Mobility Shift Assay (EMSA); the levels of TNF-α protein were measured by radio-immunoassay (RIA); the water content of rat brain was measured and pathomorphological observation was carried out. NF-κB activation, the levels of TNF-α protein and the water content of rat brain were significantly increased ($P<0.01$) following TBI in rats. Compared with Group B, NF-κB activation ($P<0.01$), the levels of TNF-α protein ($P<0.01$) and the water content of brain ($P<0.05$) began to decrease obviously after injury in Groups C and D. β-Aescin could dramatically inhibit NF-κB activation and the expression of TNF-α protein in the rat brain, alleviate rat brain edema, and that could partially be the molecular mechanism by which β-Aescin attenuates traumatic brain edema.

Key words: Brain injuries, β-Aescin, Nuclear factor-κB, Tumor necrosis factor-α, Rats
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INTRODUCTION

β-Aescin, the major active biosubstance from Aesculus hippocastanum (Hippocaslanaceae) the horse chestnut tree, has shown antioedematous efficiency for clinically significant activity in chronic venous insufficiency, haemorroids, post operative and traumatic brain edema (Liu, 1993). But the antioedematous mechanism of β-Aescin is still unclear. The aim of this study is to investigate the inhibiting effect of β-Aescin on NF-κB activation and expression of TNF-α protein in the rat brain after head injury, to elucidate the potential molecular mechanism by which β-Aescin attenuates traumatic brain edema.

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METHODS

Animals

Sixty-two Sprague-Dawley rats (purchased from Zhejiang University Experimental Animal Center), weighing 250–300 g, were randomly divided into four groups: (1) sham operated (Group A, 8 rats); (2) injured (Group B, 18 rats); (3) β-Aescin treatment (Group C, 18 rats); (4) pyrrolidine dithocarbamate (PDTC) treatment (Group D, 18 rats). All rats were anesthetized with pentobarbital sodium (50 mg/kg body weight) through intraperitoneal injection and fixed on a stereotaxic instrument. The skin of the right copular part was opened to expose the skull and a hole was drilled into the skull at the level of the parietal cortex (2 mm to the lambda, 2 mm to the fontanel and 2 mm to the sagittal suture) and the exposed dura was

kept intact. Focal brain injury was induced with a free-falling object (weighting 20 g, falling from 30 cm height) in Groups B, C and D. Group A received the same surgical procedures except that it was not subjected to lateral cortical impact injury. After brain injuries, pyrrolidine dithocarbamate (PDTC, an inhibitor of NF-κB purchased from Sigma) was administered at dose of 100 mg/kg body weight in Group D, and β-Aescin (10 mg/kg, purchased from Shandong Liuye Pharmaceutical CO. Ltd) was given intraperitoneally to Group C. Groups A and B received only an intraperitoneal injection of 0.9% saline solution (0.9% N.S). The animals were allowed to recover for periods between 6 h and 3 d and were then killed at 6 h, 24 h and 3 d respectively after injury. The brains were immediately obtained and stored at –70 °C until the time of assay.

Brain tissues

Nuclear and cytoplasmic proteins were isolated with a method described previously (Ichikawa *et al*., 1986). Briefly, brain tissue samples (about 0.5 g) were washed in 4 °C phosphate-buffered saline (PBS), then homogenized in a 1/5 volume of ice-cold 0.1 mol/L KH_2PO_4 (pH 7.4, 4 °C). The homogenates were centrifuged at 13000 g for 15 min. The supernatant was discarded; the remaining layer was dissolved in a 1/3 volume of buffer (HEPES 20, KCl 10, NaVa 0.1, DTT 1, PMSF 1, EDTA 1 mmol/L, glycerol 10%). After 10 min incubation on ice, proteins obtained were then centrifuged for 5 min at 8723 g. The nuclear pellets were dissolved in a 1/3 volume of buffer (HEPES 20, KCl 10, NaVa 0.1, DTT 1, PMSF 1, EDTA 1, NaCl 420 mmol/L, glycerol 20%) and left on ice for 20 min, then centrifuged at 10306 g for 10 min. Aliquots of nuclear extracts were stored at –20 °C and used for EMSA of NF-κB activity.

EMSA

The double-stranded DNA probes were labeled with [γ-P^{32}] ATP by T_4 polynucleotide kinase (Promega). Nuclear protein (100 μl) was incubated with poly(dI-dC) on ice for 10 min, after buffer and probes were added, it was incubated with radioactively labeled DNA probes (about 20000 cpm) for 20 min at room temperature in a 1/5 volume of binding buffer (HEPES 50, KCl 50, $MgCl_2$ 5, DTT 5, EDTA 5 mmol/L, poly(dI-dC) 250 mg/L, glycerol 25%). The nuclear proteins were mixed with loading buffer and then electrophoresed on 4% polyacrylamide gel with 0.5 mol/L TBE. Autoradiograms were developed by exposing vacuum-dried gels to X-ray film at –80 °C for 72 h.

TNF-α assay

A part of the rat brain (about 0.5 g) was excised and homogenized with a tissue homogenizer in a 1/5 volume of 0.1 mol acetic acid. After centrifugation at 3500 g for 15 min, the supernatant was collected. The above procedures were carried out again. The concentrations of TNF-α proteins in rat brain homogenates were measured with radio-immunoassay kit (purchased from Beifang Bio-Tech Institute).

Brain water content

A part of the rat brain (about 5 mm×5 mm×5 mm tissue cubes) was excised from the parietal cortex directly adjacent to the lesion core. The wet weight (WW) was rapidly measured with a chemical balance. The tissue was then dried in a desiccator oven at 100 °C for 24 h to reach constant dry weight (DW). The tissue water content was calculated as $(WW–DW)/WW$.

Histopathology

The brains were embedded in paraffin and sliced coronally throughout the lesion area. At each 60 mm level sections were taken, stained with hematoxylin and eosin and identified microscopically.

Statistical analysis

All the data were expressed in mean±*SEM*, student's *t* test and χ^2 was used to evaluate the statistical significance, which was $P<0.05$ or 0.01.

RESULTS

Pathomorphological observation

At 6 h after injury, the neurons in the lesion site had a pathologic appearance: neutrophils had considerably started to infiltrate the lesion, fibrin and blood cells extravasation had increased, and the cell body was shrunken and angulated, the cytoplasma was eosinophilic and the nucleus was pyknotic. Edema was observed near the impact site. These pathologic changes were significantly decreased in Groups C and D.

Tissue water content

In the injured group, tissue water content adjacent to the lesion site was significantly increased (P<0.01) compared to the sham operated group at 6 h, 24 h and 3 d after trauma; at 24 h after injury, tissue water content had reached its maximum and then began to reduce. Compared with the sham operated group at 6 h, 24 h and 3 d after trauma, tissue water content was not significant (P>0.05) in β-Aescin treatment group; Tissue water content in both β-Aescin and PDTC treatment group were significantly reduced (P<0.05) compared to the injured group at the same time after trauma. Compared to the sham operated group tissue water content was not significant (P>0.05) at 6 h, but had increased (P<0.05) at 24 h and 3 d after injury in the PDTC-treatment group (Table 1).

NF-κB activation and TNF-α protein concentrations

In the sham operated animals, the activation of NF-κB and the concentrations of TNF-α protein in the ipsilateral cortex were at very low levels and not statistically significant at 6 h, 24 h and 3 d after operation (P>0.05), while in the traumatic groups, they increased significantly (P<0.01), and the activation of NF-κB in the rat brain had reached its maximum at 24 h after injury. Compared to the injured groups, the activation of NF-κB and the concentrations of TNF-α protein in the rat brain began to decrease obviously after trauma in both β-Aescin and PDTC treatment groups (P<0.01), and had no significant difference (P>0.05) compared from the sham operated (Tables 2 and 3).

Table 1 The water content of rat brain in four groups (%, $\chi\pm s$)

Groups	n	Time after injury (h)		
		6	24	72
Sham operated	8	68.45±5.21	69.91±8.25	70.65±1.41
Injured	18	76.69±6.83*	89.50±5.03*	79.79±7.84*
β-Aescin	18	69.97±4.06$^{\nabla}$	70.51±1.36$^{\nabla}$	70.41±13.53$^{\nabla}$
PDTC	18	71.79±13.74$^{\nabla}$	81.63±7.73$^{**\nabla}$	77.52±10.58$^{**\nabla}$

$^{*}P$<0.01, $^{**}P$<0.05 compared with sham operated group; $^{\nabla}P$<0.05 compared with injured group

Table 2 NF-κB activation of rat brain in four groups (×10^4, $\chi\pm s$)

Groups	n	Time after injury (h)		
		6	24	72
Sham operated	8	3.0±0.11	2.46±0.83	3.83±0.79
Injured	18	25.74±2.51*	35.17±2.97*	28.31±1.32*
β-Aescin	18	4.24±1.23$^{\nabla}$	3.08±1.13$^{\nabla}$	3.37±2.49$^{\nabla}$
PDTC	18	4.01±0.36$^{\nabla}$	3.46±1.48$^{\nabla}$	4.41±5.03$^{\nabla}$

$^{*}P$<0.01 compared with sham operated group; $^{\nabla}P$<0.01 compared with injured group

Table 3 The concentrations of TNF-α protein in rat brain homogenates in four groups (ng/ml, $\chi\pm s$)

Groups	n	Time after injury (h)		
		6	24	72
Sham operated	8	40.81±12.46	39.87±8.75	39.63±5.12
Injured	18	236.63±5.32*	253.78±15.80	262.38±5.86*
β-Aescin	18	56.44±5.85$^{\nabla}$	40.11±9.52$^{\nabla}$	41.53±13.64$^{\nabla}$
PDTC	18	44.37±17.46$^{\nabla}$	45.89±6.23$^{\nabla}$	40.24±9.45$^{\nabla}$

$^{*}P$<0.01 compared with sham operated group; $^{\nabla}P$<0.01 compared with injured group

DISSCUSION

Several different pathophysiological events are thought to underlie secondary brain edema following craniocerebral trauma. These include the extracellular release of excitatory amino acid, the intracellular accumulation of calcium, generation of free radicals, and synthesis of eicosanoid products. Numerous studies have demonstrated that inflammatory reaction has an important role in secondary brain edema following brain injury. The inflammatory cytokines have been proposed to contribute to traumatic processes. The cytokine levels in the central nervous system (CNS) significantly increase after TBI, and there is strong corelationship between the levels of cytokine and the severity of brain injury (Stover *et al*., 2001). NF-κB activity may regulate much the expression of inflammatory cytokine, which can contribute to a pathologic response in TBI (Lee and Burekart, 1988). NF-κB is an intracellular transcription factor that regulates transcription of mRNA for several inflammatory mediators in various cells. NF-κB is activated by numerous stimuli, including inflammatory cytokines, reactive oxygen species (ROS) and other cytotoxic agents. Activation of NF-κB results in production of proinflammatory cytokines, potentially neurotoxic reactive oxygen species and exitotoxins, amyloid precursor protein (APP), calcium-binding proteins (CBPs), excitatory amino acid (EAA), and nitric oxide (NO). This production of gene products and proteins induced by NF-κB activation involved in inflammatory, immune and stree response (Lee and Burekart, 1988; Baeuerle and Baltimore, 1996). NF-κB in its inactive form is present in the cytosol as a three-subunit complex, with the prototypical components being P65 and P50 (transcription factor dimmer) and IκB (inhibilory subunit) (Bakdwin, 1996; Beg and Bakdwin, 1993). NF-κB is activated by signals that activate IκB kinase (IKK), resulting in phosphorylation of IκB; this targets IκB for degradation in the proteosome and frees the P65-P50 dimer, which then translocates to the nucleus and binds to consensus κB sequences in the enhancer region of κB-responsive genes (Beg *et al*., 1992). Diverse signals can induce NF-κB activation, including TNF-α, IL-1, IL-6 and IL-8. Functicial NF-κB complexes are present in essenticially all cell types in the nervous system, including neurons, astrocytes, microglia and oligodendrocytes (Korner *et al*., 1989). Nanaka *et al*.(1999) reported finding that levels of NF-κB activation in cerebral cortex are increased within hours of TBI in rats, after which they remain elevated for at least 24 h .

The result of this study demonstrated that NF-κB activation in cerenral cortex was at very low levels in the sham operated group, but increased significantly after a controlled lateral cortical impact model of TBI in rats. NF-κB activation had increased at nearly 6 h and peaked at 24 h after trauma. These results suggest that NF-κB is involved in secondary brain damage after TBI, and plays a central role in the injury-induced inflammatory response leading to secondary results.

TNF-α proteins are so-called proinflammatory cytokines, which are responsible for mediating a variety of processes in the host response to inflammatory diseases. Many studies have demonstrated that resident cells in the central nervous system are capable of TNF-α protein production. Microgliacytes and astrocytes are major sources of TNF-α proteins within the brain (Connor *et al*., 1998). It is speculated that TNF-α proteins that are increased after brain injury, play an important role in the traumatic process. TNF-α proteins can cause damage and ultimate death directly (Suzumura *et al*., 1999). TNF-α proteins are also important stimuli of NF-κB and can activate NF-κB. The induced activation of NF-κB initiated TNF-α proteins expression and TNF-α proteins have important positive feed back effect on the activation of NF-κB.

This study demonstrated that TNF-α proteins expression in the rat brain significantly increased following TBI, and at the same time water content of the rat brain also increased. After a specific inhibitor of NF-κB, PDTC was applied, the activation of NF-κB and the expression of TNF-α proteins in the rat brain were decreased markedly. The above results indicate that the dynamic changes in the activity of NF-κB parallel the changes in the expression of TNF-α proteins and the water content of the rat brain. Deregulated activation of NF-κB in the injured brain may be implicated in the development of secondary brain damage. Some studies showed injury-induced NF-κB activation was attenuated in the TNF-α receptor knockout mice and neuronal damage following

TBI was decreased (Sullivan *et al*., 1999).

β-Aescin, the major active biosubstance from Aesculus hippocastanum (Hippocaslanaceae) the horse chestnut tree, has shown antioedematous efficiency for a clinically significant activity in traumatic brain edema (Liu, 1993). But the antioedematous mechanism of β-Aescin is still unknown, specially at the molecular level. At present, the antioedematous mechanism of β-Aescin suggest: (1) β-Aescin can induce the release of adreno-corticotropin and corticosterone levels and attenuate tissue edema (Hiai *et al*., 1981). (2) β-Aescin can deregulate the expression of inflammatory cytokines. (3) β-Aescin can attenuate the breakdown of plasmalemma. (4) β-Aescin can elicit lipid peroxidation (Fransserr *et al*., 1995). This study suggested that NF-κB activation began to decrease at 6 h and was still at low levels 3 d after brain injury in β-Aescin treatment group. The expression of TNF-α proteins in the rat brain also decreased at the same time. It has been demonstrated that β-Aescin can dramatically inhibit NF-κB activation and deregulate the expression of TNF-α protein, alleviate rat brain edema, and may partially be the molecular mechanism by which β-Aescin decreases traumatic brain edema. But β-Aescin has the wide ranging mechanisms of antioedematous efficiency and other antio-tissue edema mechanisms of β-Aescin must be studied further. For example, β-Aescin can flush out free radicals, which reactive oxygen species is also an important stimuli of NF-κB. β-Aescin can also inhibit the activation of NF-κB by this mechanism. In conclusion, the inhibiting effect on NF-κB activation may partially be the molecular mechanism by which β-Aescin attenuates traumatic brain edema.

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