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The Akt/GSK-3β pathway mediates flurbiprofen-induced neuroprotection against focal cerebral ischemia/reperfusion injury in rats

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ABSTRACT

Apoptosis is one of the major mechanisms of cell death during cerebral ischemia and reperfusion injury. Flurbiprofen has been shown to reduce cerebral ischemia/reperfusion injury in both focal and global cerebral ischemia models, but the mechanism remains unclear. This study aimed to investigate the potential association between the neuroprotective effect of flurbiprofen and the apoptosis inhibiting signaling pathways, in particularly the Akt/GSK-3β pathway. A focal cerebral ischemia rat model was subjected to middle cerebral artery occlusion (MCAO) for 120 min and then treated with flurbiprofen at the onset of reperfusion. The infarct volume and the neurological deficit scores were evaluated at 24 h after reperfusion. Cell apoptosis, apoptosis-related proteins and the levels of p-Akt and p-GSK-3β in ischemic penumbra were measured using TUNEL and western blot. The results showed that administration of flurbiprofen at the doses of 5 and 10 mg/kg significantly attenuated brain ischemia/reperfusion injury, as shown by a reduction in the infarct volume, neurological deficit scores and cell apoptosis. Moreover, flurbiprofen not only inhibited the expression of Bax protein and p-GSK-3β, but also increased the expression of Bcl-2 protein, the ratio of Bcl-2/Bax as well as the P-Akt level. Taken together, these results suggest that flurbiprofen protects the brain from ischemia/reperfusion injury by reducing apoptosis and this neuroprotective effect may be partly due to the activation of Akt/GSK-3β signaling pathway.

1. Introduction

Ischemia and reperfusion (I/R) occurs in a wide range of clinical settings, including trauma, cardiac arrest, cardiac surgery, thrombolysis treatment, organ transplantation and hypovolemic shock with resuscitation. Apoptosis is one of the major mechanisms that lead to cell death after cerebral ischemia and reperfusion [1,2]. Ischemia followed by restoration of blood flow exacerbates neuronal apoptosis in the cortex and hippocampus. Cerebral ischemia and reperfusion injury could induce apoptosis and a large number of TUNEL-positive cells were observed 24 h after reperfusion [3]. Similarly, it has also been demonstrated that cerebral hypoxia could increase programmed cell death and Bax protein expression and alter the Bax/Bcl-2 protein ratios in the cerebral cortex [4].

Unlike necrotic cell death, apoptosis was an active process of cell death that requires activation of associated genes and synthesis of their encoded proteins. Activated Akt phosphorylates several downstream targets of the survival and apoptotic pathways, leading to diminished cell apoptosis including glycogen synthase kinase-3β (GSK-3β), a crucial regulator of multiple cellular functions including ischemia-induced cell death [5,6]. GSK-3β can deteriorate cell injury and increase caspase-3 activity in the hippocampal CA1 neurons after transient global ischemia [7]. GSK-3β is a constitutively active enzyme that can be inactivated by Akt via phosphorylation [6]. Moreover, the anti-apoptosis mechanisms of Akt involve inhibiting certain proteins of the Bcl-2 family. Recent studies indicate that Bcl-2 family proteins, downstream components of the PI3K/Akt signaling pathway, play a vital role in regulating cellular survival/death following hypoxia and ischemia [8]. It has been suggested that the balance between the protein levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax played an important role in regulating apoptotic cell death, i.e., increase in the cellular ratio of Bcl-2 and Bax could prevent apoptotic cell death [9]. These findings suggested that apoptotic cell death after ischemia/reperfusion could be regulated by altered expression of the Bcl-2 and Bax genes.

Flurbiprofen axetil (FA) utilizes a lipid microsphere drug delivery system, which promotes both aggregation of flurbiprofen granular at inflammatory lesion sites and absorption by inflammatory cells, resulting in effective target therapy. Flurbiprofen has been found to be highly efficacious in neuroprotection in neuro-degeneration [10] from both histological and behavioral standpoints, and it could significantly improve neurologic deficit score and mean brain infarct volume percentage under cerebral ischemic conditions [11], but little is known about the underlying molecular mechanism by

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which the neuroprotection occurs. The present study aimed to test the hypothesis that administration of flurbiprofen may provide neuroprotection in focal cerebral ischemia–reperfusion injury in a rat model of middle cerebral artery occlusion (MCAO) and this neuroprotection is associated with inhibition of apoptosis mediated by activation of the Akt/GSK-3β pathway.

2. Materials and methods

2.1. Experimental animals

Adult male Wistar rats weighing 250–310 g were obtained from the Experimental Animals Center, Shandong University. Animals were housed in a room at a controlled temperature of 25 ± 2 °C and a relative humidity of 50 ± 10% with alternating 12 h light and dark cycles and free access to food and water. All rats were acclimatized in our animal facility for at least 1 week prior to experiments.

Rats were randomly divided into five groups: sham group, ischemia/reperfusion (I/R) group, 5 mg/kg, 10 mg/kg dose of flurbiprofen axetil groups (the dosage was referred to the previous study by Salzberg-Brenhouse et al. [12]) and 1 mL/kg vehicle group (lipomicroballoons in which flurbiprofen was dissolved). All drugs were administered via tail–vein injection at the onset of reperfusion.

2.2. Focal cerebral ischemia

Transient focal cerebral ischemia was produced by the MCAO procedure as described by previously [13]. Briefly, rats were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.). The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. A 18 mm long nylon suture (ϕ: 0.2 mm) with its tip rounded by heating near a flame was introduced into the ECA lumen and advanced into the ICA until a mild resistance was felt, thereby to block the origin of the middle cerebral artery. After 120-min ischemia, reperfusion was accomplished by withdrawing the suture. The control rats received all surgical procedures but without the suture inserted.

2.3. Neurologic deficit score (NDS)

Twenty four hours after reperfusion, the neurological deficit score of each rat was obtained according to Longa's method [14] by an individual who was blinded to the experimental treatment groups. Five categories of motor neurological findings were scored: 0, no observable neurological deficit: 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling; 4, forelimb flexion, unable or difficult to ambulate.

2.4. Evaluation of ischemic infarct area

The rats were deeply anesthetized as described above and sacrificed by decapitation 24 h after reperfusion. The brains were quickly removed and cut into five 2-mm thick coronal sections and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37 °C for 20 min in the dark and photographed. Infarct brain was identified as an area of unstained tissue. The area of infarct and each hemisphere were measured by a morphological image-analysis system. Therefore, the percentage of relative infarct volume was evaluated by calculating the percentage of cerebral ischemic volume in the ipsilateral hemispheric volume (100 × total infarct volume/ipsilateral hemispheric volume).

2.5. TUNEL staining

DNA damage was assessed on paraffin-embedded sections by In Situ Cell Death Detection Kit (Roche, Germany). The sections (n = 6, in each group) were prepared using the same method as immunohistochemistry analysis at 24 h after reperfusion. The staining was performed according to the manufacturer's instructions. The cells displaying brown staining within the nucleus were counted as TUNEL-positive cells. The number of TUNEL-positive cells was determined in three microscopic eyeshots by a person blinded to the group assignment and expressed as percentage under high-power magnification (×400).

2.6. Western blot

Western blot was performed as described previously [15]. Tissues (n = 6, in each group) corresponding to the ischemic penumbra were minced into fragments, homogenized in lysis buffer (50 mmol/L Tris–HCL pH 6.8, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 0.5% NP-40 and protease inhibitor cocktail) and spinned down (2000 g, 5 min, 4 °C). The protein concentrations were determined using a BCA Protein Assay reagent kit (Pierce, Rockford, IL). The lysates were separated by 12% SDS–PAGE and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with solution of 5% skim milk powder in 1× TBS for 1 h, the membranes were incubated with primary antibodies of anti-p-Akt, anti-Bcl-2, anti-bax (1:400 dilution, Santa Cruz, CA, USA), and anti-GSK-3β (1:1000 dilution, Cell Signaling, Beverly, MA, USA) overnight at 4 °C, respectively. After washing three times with TBS-T for 5 min, the blot was incubated with horseradish peroxidase-labeled second antibodies of anti-mouse IgG or anti-rabbit IgG (1:6000 dilution, ProteinTech Group, Chicago, IL, USA), respectively. The blot was again washed three times with TBS-T before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL). An enhanced chemiluminescent (ECL) detection system was used according to the manufacturer's protocol, and immunoblots were exposed to autoradiography film (Hyperfilm-ECL, Amersham Pharmacia Biotech). To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein GAPDH.

2.7. Statistical analysis

All analyses were performed using SPSS v11.0 (SPSS Inc., Chicago, IL). All data were represented as means ± SD. The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to determine where differences among groups existed. Differences were considered statistically significant for values of $P < 0.05$.

3. Results

3.1. Flurbiprofen attenuated neurologic deficit scores and infarction volumes

In ischemia/reperfusion group, the infarct volume was 41.6 ± 5.4% after middle carotid artery occlusion for 2 h followed by reperfusion for 24 h. However, the occlusion for 2 h followed by reperfusion for 24 h resulted in an infarct volume of 28.7 ± 5.0% and 21.8 ± 3.5% in doses of flurbiprofen 5 mg/kg and 10 mg/kg groups, respectively. The infarct volumes in these two groups were significantly smaller than those of ischemia/reperfusion and vehicle groups ($P < 0.01$). Furthermore, there was a significant difference in infarct volume between 5 mg/kg and 10 mg/kg