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Using a novel *in vivo* model to study the function of nuclear factor kappa B in cerebral ischemic injury

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Summary

Background: Cerebral ischemia is a situation with a deficit blood supply to the brain, which eventually leads to cell death, inflammation, and tissue damage. Nuclear factor kappa B (NF-κB) plays an important role in inflammation and immune regulation. The aim of this study was to test the function of the activation of NF-κB *in vivo* in cerebral ischemic injury.

Material/Methods: We generated an animal model that used the method of occlusion of the middle cerebral artery (MCAO). The 60 traits were equally divided into 5 groups to investigate the role of NAC pretreatment: (1) sham-operation (control), (2) ischemia for 6 hours, (3) ischemia for 6 hours and NAC pretreatment, (4) ischemia for 24 hours, (5) ischemia for 24 hours and NAC pretreatment. The 36 rats were divided randomly into 3 groups: (A) recombinant adenovirus expressing wild-type κBα (AdIκBαM) group, (B) recombinant adenovirus expressing wild-type IκBα (AdIκBα) group, and (C) simple ischemia group. Triphenyltetrazolium chloride (TTC) was used to measure infarct volume. Detection of expression of NF-κB was by Immunohistochemistry analysis.

Results: The infarct size of the 24-hours ischemia groups were bigger than those of 6-hours ischemia groups ($P<0.01$). The infarct size of using NAC pretreatment groups was obviously reduced compared with saline control groups ($P<0.01$).The percentage of cortical p65-positive cells of the group of (A) were significantly less than the groups of (B) and (C).

Conclusions: Our data suggest that N-acetylcysteine (NAC) and Ad-IκBα-Mut can inhibit the activation of NF-κB *in vivo*, reduce the focal infarct size, and protect the brain tissue in ischemia.

key words: **NF-κB • cerebral ischemia • neurionoprotect • adenovirus • inflammation**

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Background

Cerebral ischemia occurs when the blood supply to the brain is deficient due to arterial obstruction or systemic hypoperfusion. The deprivation of oxygen and glucose in the ischemic brain eventually leads to cell death, inflammation, and tissue damage [1,2]. The risk of stroke is estimated at 10–20% in the 90 days after a transient ischemic attack [3]. In recency years, some studies have provided excellent methods for stroke prevention [4]; however, the molecular mechanism underlying cerebral ischemic injury is still poorly understood.

NF-κB plays an important role in inflammation and immune regulation [6]. Recent expression profile studies of damaged brain tissue have identified hundreds of genes that are upregulated, including NF-κB [5]. NF-κB is a family of transcription factors which commonly consists of the heterodimer of a 65 kDa protein (p65) and a 50 kDa protein (p50) that are sequestered in the cytoplasm by an anchor protein called inhibitor of NF-κB (IκB) [5,6]. The activation of NF-κB is tightly controlled by IκB, which binds to the cytoplasmic NF-κB complexes [7,8]. NF-κB activation is thought to play an important role in cerebral ischemic injury, but the exact mechanism remains controversial. Some reports have demonstrated that asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, increases the activity of NF-κB [9], and activation of NF-κB is able to prevent cerebral ischemic injury [10,11], whereas other reports have shown less consequence in the NF-κB-deficient animal models [12,13].

Phosphorylation of IκB on serines 32 and 36 by IκB kinase leads to its ubiquitination and degradation by proteosomal enzymes. This allows the NF-κB heterodimer to activate and translocate into the nucleus, regulating gene expression [14]. Substitution of serines 32 and 36 of IκB by alanines postpones IκB degradation [15, 16]. Accordingly, overexpression of mutated IκB (denoted IκBα-Mut) prevents NF-κB activation in transiently or stably transfected cells [15,17] and in transgenic mice [18].

Here, we generated a unique animal model with transient expression of wild-type and mutant IκB *in vivo*. We then combined this model with the cerebral ischemic injury animal model and successfully tested the function of the activation of NF-κB *in vivo* in cerebral ischemic injury. We demonstrated that inhibition of NF-κB activation *in vivo* is capable of preventing cerebral ischemic injury.

Material and Methods

Animals

All the animal experiments were performed in the animal facility of our institute with approved protocol (SYXX20020017).

Reagents

One hundred and twenty 2-month-old male Wistar rats were used in this study. Sixty rats were selected randomly from the total 120 Wister rats and were equally divided into 5 groups to investigate the role of NAC pretreatment: (1) sham-operation (control), (2) ischemia for 6 hours, (3) ischemia for 6 hours and NAC pretreatment, (4) ischemia for 24 hours, and (5) ischemia for 24 hours and NAC pretreatment. Simultaneously, 24 rats were randomly selected and were divided into 2 groups for cortical injection of recombinant adenovirus-expressed IκB (Ad-IκBα) and its mutant (Ad-IκBα-Mut), respectively (n=12). The other 36 rats were divided randomly into 3 groups: (A) AdIκBαM group, (B) AdIκBα group, and (C) simple ischemia group.

N-acetylcysteine (Sigma), NF-κBp 65 polyclonal antibody (Santa Cruz), PV6001 Immunohistochemistry Detection (Zhong Shan Co) and *In Situ* Cell Death Detection (Boehringer Mannheim) were used in this study. Recombinant adenovirus AdIκBαM (mutation on serines 32 and 36 of IκBα gene) and Ad-IκBα (containing wild-type IκBα gene) were constructed and provided by Dr. Bingrong Liu at our institute [19]. Concentration of adenovirus was 2.5×10^{12} particles/ml.

Animal model of middle cerebral artery occlusion (MCAO)

We used the method of MCAO adapted from Longa et al. [5]. Briefly, the rats were anesthetized with 10% chloral hydrate, 0.4g/kg i.p. The right common carotid artery (CCA), the right external carotid artery (ECA) and the right internal carotid artery (ICA) were isolated. The CCA and the ECA were tied permanently. The end-tips of the 0.165 mm nylon suture were burned with a flame. A microaneurysm clamp was applied to the ICA. Close to the CCA bifurcation and through a small opening in the CCA, the nylon suture was inserted into the CCA. The silk suture around the CCA was tightened and then the microaneurysm clamp was removed. The nylon suture was guided in the ICA up to the origin of the MCA. The length from the CCA bifurcation to the origin of the MCA was about 18.5±0.5 mm and the length in the Sham operated group is less than 15 mm.

The ischemia group animals were killed after 6 h or 24 h of occluding the middle cerebral artery. One group of animals was given with NAC in a dosage of 150 mg/kg 30 min before occlusion. Control rats received the same volume of saline solution.

Cortical injection of recombinant adenovirus.

Cortical injection of recombinant adenoviruses expressed with AdIκBαM and AdIκBα was carried out using a stereotaxic instrument. Each rat was subjected to 4 cortical injections in the following locations: (1) 1 mm caudal to the Bregma, 4.6 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull; (2) 2 mm caudal to the Bregma, 4.3 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull; (3) 3 mm caudal to the Bregma, 4.6 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull; and (4) 4 mm caudal to the Bregma, 5.2 mm lateral to the midline of the skull, and 4 mm ventral to the exterior surface of the skull. All the target points were in the right side of the brain (ie, the ipsilateral hemisphere) to the MCAO. Two microliters of adenoviral suspensions containing 1×10^{11} particles/ml were injected into each point at a rate of 2.0 μl/min. The needle was withdrawn after a

course of 10 min. Recombinant adenovirus with AdIκBαM, or AdIκBα and the same volume of saline solution were injected at 48 h before occlusion.

The Zea Longa's neurological deficit score was based on a 5-point scale [20]: 0 = no neurological deficit; 1 = mild focal neurological deficit (failure to extend left forepaw fully); 2 = moderate focal neurological deficit (circling to the left), and 3 = severe focal neurological deficit (falling to the left); rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. The rats with scores of 1 to 3 were chosen for this study.

Detection of expression of IκBαM

Immunohistochemical analysis with antibody against IκB and fluorescence microscopy analysis by observing GFP/IκBαM was performed. The expression of IκBαM in the infected cortical region was successively detected at day 1 to day 6 after AdIκBαM injection. Brown coloring of nuclei was used as the positive signal of IκBαM staining of immunohistochemical analysis.

Measurement of infarct volume

After killing the rats, their brains were removed. The cerebrums were cut into 2 mm-thick coronal blocks for a total of 6 to 7 blocks in each rat. The sections were immersed in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 min, avoiding light. The posterior surface of each section was photographed. The total area and the colorless area of each section were measured by an image analysis system (Motic Images Advanced 3.0). The infarct volume was calculated with the following formula: $V_{total\ volume}$ = t(A1+A2+…+An)-t(A1+An)/2 (t: thickness of each section, A1~An: area of each section). $V_{infarct\ volume}$ = t(A1+A2+…+An)-t(A1+An)/2 (A1~An: each infarct area). The percentages of infarct volumes were also calculated ($V_{infarct\ volume}$ /$V_{total\ volume}$ ×100%).

Fixation of samples

After the rat was sacrificed, its chest was opened to rapidly expose the heart. Through the left ventricle a soft tube was inserted into the aorta ascendens and the right auricle was cut. One hundred millimeters of normal saline was injected into the aorta ascendens, and then 100 ml of 40 g/L paraformaldehyde was slowly injected. Then the brains of rats were removed and were placed in paraformaldehyde solution for 24 hours, and they were cut into 3 mm-thick coronal blocks and dehydrated through gradient alcohol and xylene. Brains were embedded by paraffin and then sliced into sections of 5 μm thick used for immunohistochemistry analysis and for terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end-labeling (TUNEL) staining.

Detection of expression of NF-κB p65 by immunohistochemistry

The tissue sections were dewaxed with xylene and rehydrated. In 0.01 mol/L citrate buffer solution, pH6.0, the sections were repaired by high temperature and high pressure, and then washed 3 times with PBS for 3 min each time. The sections were incubated with 3% H_2O_2 for 10 min, washed with PBS 3 times for 3 min. Rabbit antibody against NF-κB p65 was added to the sections at a ratio of 1:100, and incubated at 4°C overnight. The sections were washed with PBS 3 times for 3 min. Goat antibody against rabbit (IgG)-HRP polymer was added into the sections and was incubated for 20 min at room temperature, and then they were washed again. The sections were stained with DAB, and then washed with tap water. Afterwards, they were counter-stained with hematoxylin. One hundred cells were counted in each field of vision under ×200 microscopic resolution (10 different fields of vision in each section). The NF-κB p65-positive cells were counted.

TUNEL staining for apoptotic cells

Sections were dewaxed with xylene and rehydrated. Then they were incubated with 3% H_2O_2 for 20 min at room temperature and washed 3 times with PBS for 3 min. Afterwards, they were incubated with proteinase K for 15 min at room temperature and washed 3 times with PBS for 3 min. A 50μl TUNEL solution was added for 1 h at 37°C and washed 3 times with PBS for 3 min. A 50 μl converter-POD solution was added to each section and incubated at 37°C for 30 min and washed 3 times with PBS for 3 min. The sections were stained with DAB and counter-stained with hematoxylin; dehydration, transparency and obstruction glass piece were performed. They were observed through a high-power microscope (×400), and the nuclei of apoptotic cells were brown. The percentage of TUNEL-positive cells was calculated (the count of TUNEL-positive cells/the count of total cells ×100%).

Statistical analysis

Statistical analysis was performed with SPSS11.5. All data are expressed as means ± s ($\bar{\chi}\pm s$). Statistical comparison used analysis of variance of completely random design. LSD-test was used to detect the difference between the 2 groups. $P<0.05$ was considered to be statistically significant.

RESULTS

Generation of animals with transient expression of wild-type and mutant IκBα

Diffused distribution of green fluorescence in brain tissues adjacent to the needle trace were observed by fluorescent microscopy 1 day after Ad-IκBα-MUT injection. Green fluorescence became much stronger 2 or 3 days after injection (Figure 1), and almost no green fluorescence could be found 6 days after injection. By contrast, only a weak basal immunoreactive signal was detected in the wild-type Ad-IκBα-injected rats (Figure 1).

To verify the specificity of IκBα expression in the generated animals, we used specific antibody against IκBα to detect the expression of IκBα in tissue sections of animals. As shown in Figure 2, the expression of IκBα-Mut in brain tissue following Ad-IκBαMut injection was strongly positive by immunohistochemistry at 2 and 3 days after Ad-IκBα-Mut injection.

Inhibition of NF-κB p65 Activity by chemical inhibitor NAC and mutant IκB *in vivo*

The sham operated group showed that NF-κB p65 is mainly in the cytoplasm, and is not expressed in nuclei. In 6-hr and

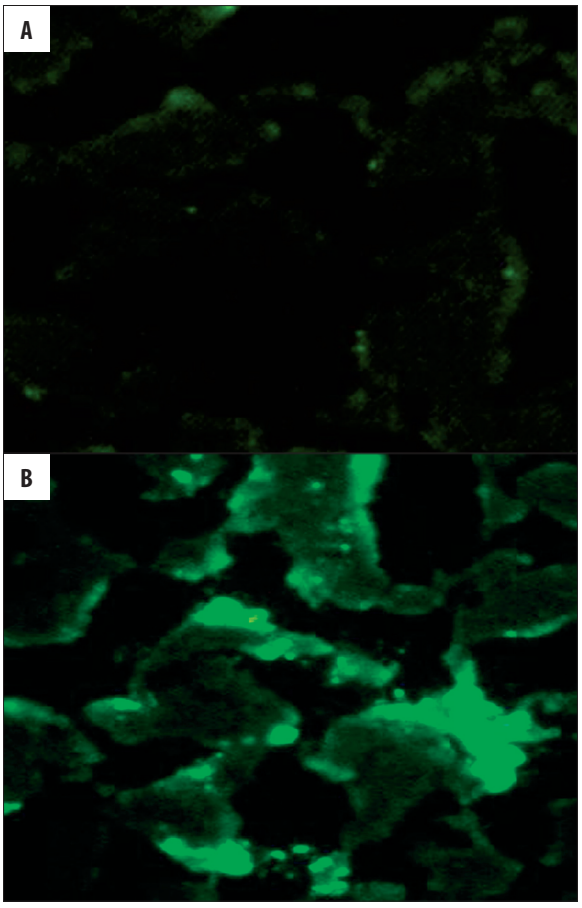

Figure 1. Generation of animals with transient expression of wildtype and mutant IκBα. Mutant IκBα (IκBα-Mut) demonstrates increasing IκBα's expression and activity. Observation of green flurescence (GFP) expressed by Ad-IvBα (**A**) and Ad-IκBα-Mut (**B**) in brain tissue adjacent to the needle-traces two days after cortical Ad-IκBα-Mut injection (×400).

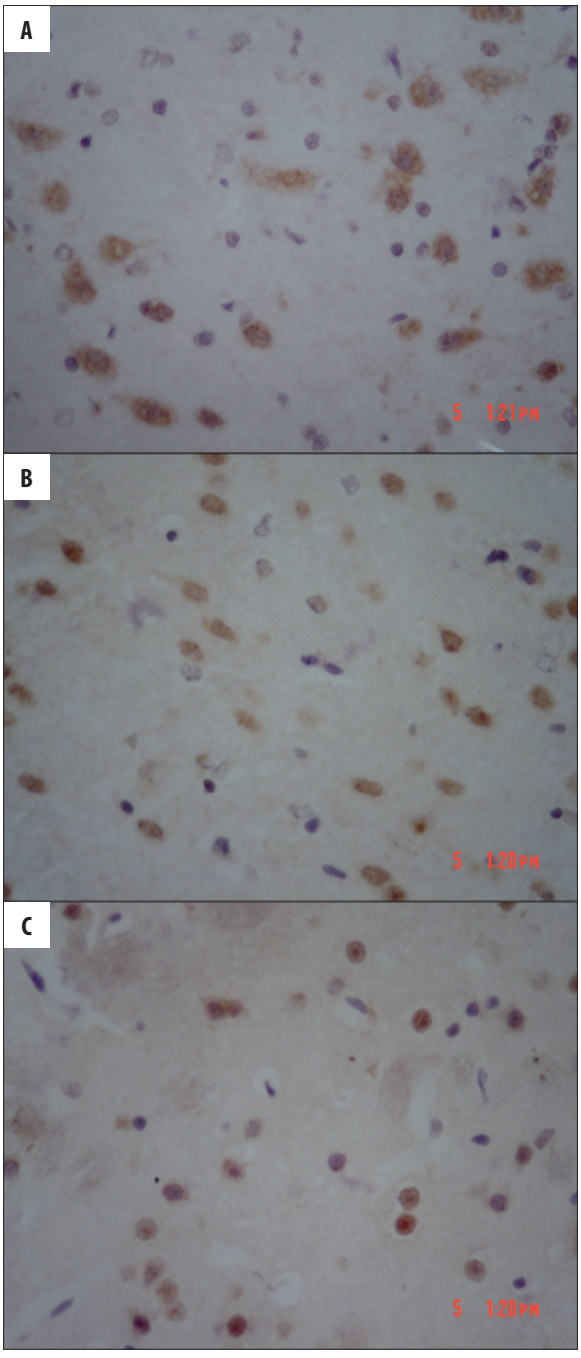

Figure 2. Mutant IκB (IκBα-Mut) prolongs IκB's expression and activity. Immunohistochemistry staining with antibody against IκB is able to detect the expression of mutant IκBα, showing brown coloring of positive immunoreaction in brain tissue adjacent to the needle-traces one day (**A**) and two days (**B**) after cortical Ad-IκBα-Mut injection (×100).

24-hr ischemia groups, NF-κB apparently translocated from the cytoplasm to the nuclei. The positive cells whose nuclei were stained were mainly in the ischemic penumbra. On the other hand, in the center of the ischemic area, the positive cells decreased and most of cells were stained in the cytoplasm. From the cell classification, we could see that positive cells were mainly neurons. There were few astrocytes and microgliocytes. As Table 1 shows, there were fewer NF-κB p65-positive cells in the groups that used NAC than in the groups that used saline as control (P<0.01, Figure 3). The percentage of NF-κB p65-positive cells in cortical fields of Ad-IκBα-Mut injection groups were significantly less than in the groups of wild-type Ad-IκBα injection and simple ischemia (P<0.01). However, there was no difference in the percentage of positive cells between groups with Ad-IκBα injection and simple ischemia (P>0.05, Table 2).

Inhibition of NF-κB *in vivo* prevents the cerebral ischemic injury

In total, 113 rats were used in this study and all data were statistically analyzed without losses. In the test of NAC pretreatment, the sham-operated group had no infarctions. The infarct size of the 24-hr ischemia groups were bigger than those of the 6-hr ischemia groups (P<0.01). The infarct size of groups using NAC pretreatment was obviously reduced compared with saline control groups (P<0.05, Table 1).

In the test of cortical adenovirus injection, compared with adenovirus Ad-IκBα groups and simple focal ischemia groups, the infarct size of Ad-IκBα-Mut injection groups decreased significantly (P<0.01). However, the difference in percentage of infarct volume between Ad-IκBα injection

Table 1. NAC prevents cerebral ischemic injury. The infarct size of 24 hours ischemia groups were bigger than those of 6 hours ischemia groups ($P<0.01$). The infarct size of using NAC pretreatment groups obviously reduced compared with saline control groups ($P<0.01$).

Groups	p65 positive cells	Apoptotic cells	Cerebral infarct volume
Ischemia 24 h + NAC	0.438±0.017*	0.346±0.028*	20.15±7.26*
Ischemia 6 h + NAC	0.455±0.020*	0.332±0.031*	8.12±2.50*
Ischemia 24 h	0.541±0.039**	0.554±0.029**	27.57±3.41**
Ischemia 6 h	0.543±0.022	0.569±0.022	14.03±4.42
Sham-operation	0.097±0.008	–	–

* Compared with saline control $P<0.01$,**compared with 6 hours ischemia groups $P<0.01$.

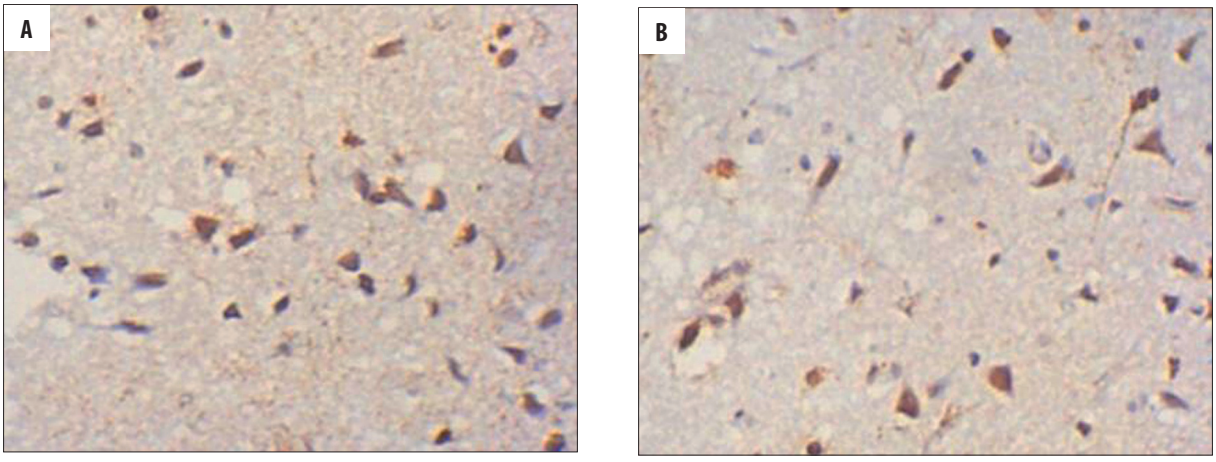

Figure 3. A NF-κB inhibitor NAC prevents the cerebral ischemic injury. Immunohistochemistry staining with the antibody against NF-κB p65 for sham-operated groups (control), 24 h ischemia groups, and 24 h ischemia with NAC treatment groups (×400).

Table 2. The ad-IκBα-Mut prevents cerebral ischemic injury. Compared with group A and group B, C, $P<0.01$. Compared with group B and C, $P>0.05$.

Groups	p65 positive cells	Apoptotic cells	Cerebral infarct volume
A. ad-IκBα-Mut	0.418±0.041	0.306±0.051	7.56±2.82
B. ad-IκBα	0.572±0.035*,**	0.512±0.071*,**	15.73±3.62*,**
C. control	0.554±0.048*	0.543±0.067*	16.34±5.17*

* Compared with A $P<0.01$, ** compared with C $P>0.05$.

groups and simple focal ischemia groups was not significant (P>0.05, Table 2).

Inhibition of NF-κB p65 prevents apoptosis

We then explored cell apoptosis after treatment with NF-κB inhibitors to gain insight into the putative mechanism leading to the prevention of cerebral ischemic injury. There were no apoptotic cells in the sham-operated group. The nuclei of apoptotic cells in ischemic and reperfusion groups were brown; most of them were neurons, located mainly around ischemic areas, and some apoptotic cells were found in the center of ischemic zones. Through ×400 microscopic resolution, we could see late apoptotic cells with small bodies and altered shapes. The nucleus was pyknotic and was divided into several parts. Apoptotic bodies were also present. There were fewer apoptotic cells in the NAC groups compared with saline groups (Table 1). The percentage of TUNEL-positive cells in cortical fields of Ad-IκBα-Mut injection groups were significantly lower than in wild-type Ad-IκBα injection and simple ischemia groups (P<0.01). However, there was no difference in the percentage of positive cells between wild-type Ad-IκBα injection and simple ischemia groups (P>0.05, Table 2).

TUNEL staining showed that apoptotic cells were mainly in the ischemic penumbra, the same as in the NF-κB-positive cells. In the ischemic center, NF-κB was mainly in the cytoplasm.

Discussion

Because mutant IκB (IκBα-MUT) prolongs IκB's expression and activity, we generated an in vivo model to express

wild-type and mutant IκB to test NF-κB function in cerebral ischemic injury. The plasmids of adenovirus with expression of mutant (Ad-IκBα-MUT) and wild-type IκBα (Ad-IκBα) were generated as described in the Methods section. Plasmids contained the green fluorescent protein (GFP) gene; therefore, infection of adenovirus was evaluated by observing the expression of GFP. Therefore, recombinant adenovirus effectively infected brain tissues and expressed IκBα-Mut protein. Because IκB is an inhibitor of NF-κB, the prolongation of IκB activity from mutant IκBα-Mut presumably will inhibit NF-κB activation longer than wild-type IκB.

We found that the percentage of cortical p65-positive cells of Ad-IκBαMut injection groups were significantly lower than in the Ad-IκBα injection and simple ischemia groups, demonstrating that the activation of NF-κB was inhibited by Ad-IκBαMut. The infarct size of Ad-IκBα-Mut injection groups was significantly reduced compared to Ad-IκBα groups and simple ischemia groups, indicating that Ad-IκBα-Mut can reduce the cerebral infarct area.

Antioxidant NAC is a precursor of glutathione and can increase the amount of glutathione within the cells, stabilize the cell membranes, and protect the activity of cells. It can also directly delete reactive oxygen species (ROS), and prevent the expression of adhesion molecules and the immersion of neutrophilic granulocytes. NAC is also a chemical inhibitor of NF-κB. We therefore administered this chemical in vivo to study the function NF-κB in cerebral ischemic injury.

From the results of our test, we found that p65 has low levels of activity in normal brain tissue. The nuclear localization apparently increased in cerebral ischemia groups compared with the sham-operated group, demonstrating that cerebral ischemia can lead to the activation of NF-κB. Injecting NAC decreased the activation of p65 and reduced the infarct area.

The above findings indicate a close relationship between NF-κB and neurocyte apoptosis [21,22]. NAC, an inhibitor of NF-κB, is potent because it can decrease number of apoptotic cells and reduce infarct area. The infarct zones in 24-hr ischemia groups were larger than in 6-hr ischemia groups. However, the activation of NF-κB p65 did not noticeably increase, indicating that in addition to NF-κB activation there are other reasons affecting the process of cerebral ischemia [23–25].

In summary, we have generated a unique animal model with transient expression of wild-type and mutant IκB to inhibit NF-κB activation. By combining this model with the cerebral ischemic injury animal model, we successfully tested the function of NF-kB activation in vivo in cerebral ischemia. Our data suggest that NAC and Ad-IκBα-Mut can inhibit the activation of NF-κB *in vivo*, reduce focal infarct size, and protect brain tissue in ischemia. Therefore, we optimistically predict that inhibition of NF-κB activation will become an effective strategy for the development of novel treatments for, or prevention of, cerebral ischemic injury.

Conclusions

In summary, we have generated a unique animal model with transient expression of wild-type and mutant IκB to inhibit NF-κB activation. By combining this model with the cerebral ischemic injury animal model, we successfully tested the function of the NF-κB activation *in vivo* in cerebral ischemia. Our data suggest that NAC and Ad-IκBα-Mut can inhibit the activation of NF-κB *in vivo*, reduce the focal infarct size, and protect the brain tissue in ischemia. Therefore, our results let us optimistically predict that inhibition of NF-κB activation might be an effective strategy for the development of novel treatments or even prevention methods for cerebral ischemic injury.

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