Antioxidants attenuate reperfusion injury after global brain ischemia through inhibiting nuclear factor-kappa B activity in rats[1]

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ABSTRACT

AIM: To investigate the effects of two antioxidants on the alterations of nuclear factor kappaB (NF-κB) activity and p65, p50 protein expression and phosphorylation of IκBα in rat hippocampus following global brain ischemia. **METHODS:** Using a 4-vessel occlusion (4-VO) as brain ischemia model, NF-κB protein (p65 or p50 subunit) expression was examined by Western blot analysis, and NF-κB activity was assayed by electrophoretic mobility shift assay (EMSA), and neuronal loss was observed by histology. **RESULTS:** NF-κB activity displayed a time-dependent manner, and p65, p50 proteins showed their peak levels after ischemia/reperfusion 6 h. NF-κB inductions (p65: 4.79±0.78, p50: 5.50±0.33, sham control=1) and activity (4.93±0.95) after 6 h of reperfusion were markedly reduced by pretreatment with antioxidants pyrrolidine dithiocarbamate (PDTC, 200 mg/kg) (p65: 1.11±0.74, p50: 1.38±0.98, activity: 2.20±0.86, respectively) or *N*-acetylcysteine (NAC, 300 mg/kg) (p65: 0.64±0.39, p50: 1.89±0.87, activity: 0.61±0.65), and histological observations of the pyramidal layer of CA1 also showed a reduction of neuronal loss in rat hippocampus (70 %±5 % or 92 %±4 % cells are survival, respectively). Furthermore, PDTC and NAC prevented the decrease (from 0.50±0.10 to 0.80±0.20 or 1.20±0.24, respectively) and phosphorylation (from 2.00±0.15 to 0.46±0.10 or 0.41±0.10, respectively) of IκBα protein in the cytoplasm. **CONCLUSION:** The protective effects of antioxidants against ischemia/reperfusion-induced injury may be mediated by down-regulation of NF-κB activity. NF-κB activation and deactivation are controlled mainly through phosphorylation and degradation of IκBκα following brain ischemia.

INTRODUCTION

NF-κB is a family of hetero- or homodimer proteins with DNA-binding and transcription activation. Inactive NF-κB is located in the cytoplasm mainly binding by inhibitory protein IκBα. When cells are exposed to stimuli, different regulatory kinases[1] result in serine phosphorylation of IκBα[2] and subsequent degradation. NF-κB thus translocates to the nucleus and activates the transcription of genes related to immune and stress response.

NF-κB was the first eurkaryotic transcription factor shown to respond directly to reactive oxygen species (ROS)[3]. At present it is still under discussion that NF-κB acts as either protective or degenerative func-

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tion[4]. Results of our previous studies[5,6] and some other investigations suggest that NF-κB plays a pro-apoptotic role in ischemic models or cultured cell models. To further investigate the relationship between ROS and NF-κB, we examined the two important antioxidants of PDTC and NAC following ischemia/reperfusion (I/R)[7].

In this study, we examined the time course of p65, p50 proteins and NF-κB DNA binding activity after I/R different time points in hippocampus of rats. To delineate the role of NF-κB in ROS-induced neuronal apoptosis *in vivo*, we attempted to determine whether PDTC and NAC would modify the NF-κB response to ischemia. To further determine the role of NF-κB in neuronal death, Cresyl violet staining was performed to examine the survival of hippocampal CA1 neurons. We also detected the alterations of IκBα and pIκBα by Western blot analysis of homogenates from cytosolic extracts of rat hippocampus.

MATERIALS AND METHODS

Materials Rabbit polyclonal anti-p65 (sc-372), anti-p50 (sc-7178), mouse monoclonal anti-IκBα (sc-1643), anti-pIκBα (sc-8404) antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Alkalline phosphatase conjugated goat anti-rabbit IgG, and goat anti-mouse IgG, and PDTC, and NAC were all from sigma (St Louis, MO, USA). 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) were from Promega (Madison, WI, USA).

Induction of ischemia Adult male Sprague-Dawley (SD) rats (purchased from Sippr-BK Experimental Animal Ltd Co, Shanghai, Grade II, Certificate No D52) weighing 250-300 g were used. Brain ischemia was induced by 4-VO, as described before by Pulsinelli *et al*[8] and by us[9]. Briefly, under anesthesia with chloral hydrate (350 mg/kg, ip), vertebral arteries were electrocauterized and common carotid arteries were exposed. On the next day, ischemia was induced by occluding bilateral vertebral arteries with aneurysm clips for 30 min. During the ischemia, animals were required to match the following criteria: completely flat electroencephalogram (EEG), maintenance of dilated pupils, absence of a cornea reflex when exposed to strong light stimulation, and maintenance of rectal temperature at (37±5) ºC. Animals not matching these criteria or with seizures were all discarded. Control animals received the same surgical procedures except bilateral carotid arteries were not occluded.

Brain tissues and drug treatment Cytosolic proteins were extracted by some modifications of a previously described procedure[10]. Briefly, tissue samples were homogenized in ice-cold buffer A (HEPES 10, $MgCl_2$ 0.5, KCl 10, edetic acid 0.1, egtazic acid 0.1, NaF 50, dithiothreitol (DTT) 1, β-phosphoglycerol 30, Na_3VO_4 1, benzamidine 1, phenylmethylsulfonyl fluoride (PMSF) 0.5, *p*-nitrophenyl phosphate (PNPP) 1 mmol/L and aprotinin 10, leupeptin 10, pepstatin A 10 mg/L; pH 7.9). Proteins were left for 10 min, after addition of 90 μL NP-40 (10 %), the homogenates were vortexed for 30 s and then centrifuged for 15 min at 800×*g*. Supernatants were used for Western blot analysis, and aliquoted, stored at -80 ºC until use.

The nuclear pellets were dissolved in buffer B (HEPES 20, NaCl 420, $MgCl_2$ 0.5, edetic acid 1, egtazic acid 1, DTT 1 mmol/L, glycerol 20 %, and enzyme inhibitors above, pH 7.9), and then were left for 30 min at 4 ºC with constant agitation. After centrifugation for 15 min at 12 000×*g*, nuclear extracts were aliquoted, and stored at -80 ºC until use. Nuclear extracts were used for Western blot analysis of p65, p50 protein expression and for EMSA of NF-κB activity.

To evaluate the effects of antioxidants blockade on ischemia-induced alterations of NF-κB, animals were given PDTC (50, 100, or 200 mg/kg, ip), NAC (100, 200, or 300 mg/kg, ip), respectively by abdominal injections 20 min before 30 min ischemia. Control rats were received vehicle (0.9 % NaCl, ip).

Western blot Western blot analysis was performed as described previously[11] with some modifications. Protein concentrations were determined using Lowry method[9]. Samples were mixed with loading buffer and boiled for 5 min. Proteins were separated on 10 % SDS-PAGE gel, then were electroblotted onto NC membranes (Millipore, Bedford, MA). After blocking for 2 h in PBS with 0.1 % Tween 20 (PBST) and 3 % BSA, membranes were incubated overnight at 4 ºC with primary antibodies. Membranes were then washed and incubated with secondary antibody for 2 h and detected by NBT/BCIP assay kit.

Electrophoretic mobility shift assay (EMSA) As previously described[11] with some modifications, double-stranded DNA probes were labeled with γ-[^{32}P] ATP by T4 polynucleotide kinase (Promega). Nuclear protein (15 μg) were incubated with the poly(dI·dC) on ice for 10 min, the buffer and probes were added and incubated with radioactively labeled DNA probes (about

20 000 cpm) for 20 min at room temperature in a 1/5 volume of binding buffer (HEPES 50, $MgCl_2$ 5, EDTA 5, DTT 5, KCl 50 mmol/L, poly(dI·dC) 250 mg/L and glycerol 25 %, pH 7.5). Nuclear proteins were mixed with loading buffer and then electrophoresed on 4 % polyacrylamide gel with 0.5×TBE. Autoradiograms were developed by exposing vacuum-dried gels to x-ray film at -80 ºC for 20-48 h.

The specificity of NF-kB binding was confirmed by adding 100-fold excess of unlabeled NF-κB or SP1 DNA probe to the assay.

Histology Rats were perfusion-fixed with 4 % paraformaldehyde 48 h after 30 min ischemia under anesthesia, exactly as previously described[9]. Paraffin sections (5 mm) were prepared and stained with Cresyl violet. The sections were examined with a light microscopy (×400) and the number of the surviving hippocampal CA1 pyramidal cells per 1 mm length was counted as neuronal density.

Statistical analysis Western blot and autoradiographic results were semiquantitatively evaluated by means of an image analyzer (LabWorks Software, UVP upland, CA). Values were expressed as mean±SD from 3-6 independent rats. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the Duncan's new multiple range method or Newman-Keuls test and $P<0.05$ were considered significant.

RESULTS

The protective effects of antioxidants PDTC and NAC against I/R injury Cresyl violet staining was used to examine the survival of hippocampal CA1 neurons. Normal CA1 pyramidal cells showed round and pale stained nuclei in cresyl violet staining (Fig 1A). The shrunken cells with pyknotic nuclei after ischemia were counted as dead cells. I/R 48 h following 30 min ischemia induced severe cell death (95 %±4 % cells are dead, Fig 1B). The number of the surviving hippocampal CA1 pyramidal cells was significantly increased as compared with I/R 48 h rats after pretreatment with antioxidants of PDTC200 (200 mg/kg) or NAC300 (300 mg/kg) (70 %±5 % or 92 %±4 % cells are survival, respectively, Fig 1C, D).

Time course of ischemia-induced activation of NF-κB in rat hippocampus after I/R Western blot and EMSA have been used to determine the status of NF-κB complex in hippocampus[5]. Western blot analysis of homogenates prepared from nuclear extracts of hippocampus was performed with anti-p65 and p50 antibodies. Time course of I/R induced changes of NF-κB was measured after rats were subjected to various periods of I/R (Fig 2A). NF-κB subunits p65 and p50 were increased evidently after transient cerebral ischemia and reached their peak levels after 6 h reperfusion, then decreased again. Fig 2B shows the time course of an EMSA from the same nuclear extracts as analysis of Western blot, which reveal parallel results as above. There was a transient, about 7-fold increment at 6 h as compared with control rats, in the levels of NF-κB p65 and p50 subunits or NF-κB DNA binding activity.

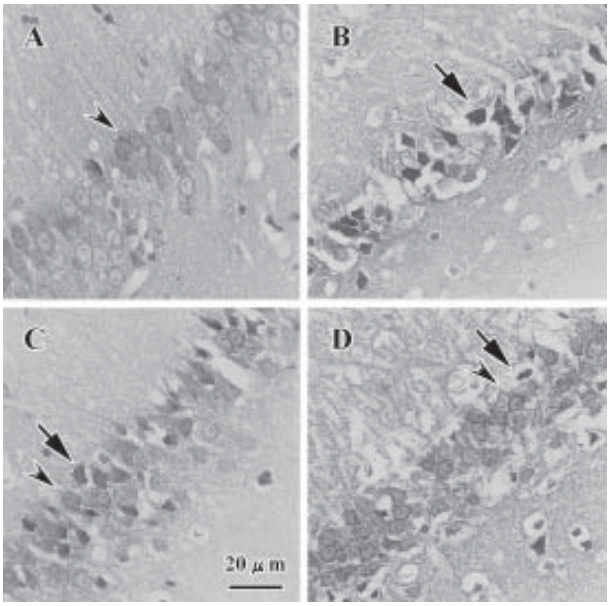

Fig 1. Effects of PDTC and NAC on 48 h I/R induced cell death. The number of surviving CA1 pyramidal cells (arrowheads) were counted from a light microscope (×400) from six independent animals (n=6). A: Control rats with normal cells (arrows). B: Rats with 30 min ischemia following a 48-h reperfusion. C: Pretreatment with PDTC (200 mg/kg) and a reperfusion for 48 h. D: Pretreatment with NAC (300 mg/kg) and a reperfusion for 48 h.

Effects of antioxidants PDTC and NAC on ischemic-induced alterations of NF-κB in rat hippocampus after I/R 6 h To verify whether activation of NF-κB after ischemia was associated with ROS, we intraperitoneally injected 4-VO rats with PDTC and NAC. As shown in Fig 3, the ischemia-induced increase of NF-κB binding activity in rat hippocampus of nuclear extracts was significantly inhibited by pretreatment with NAC (100-300 mg/kg) in a dose-dependent

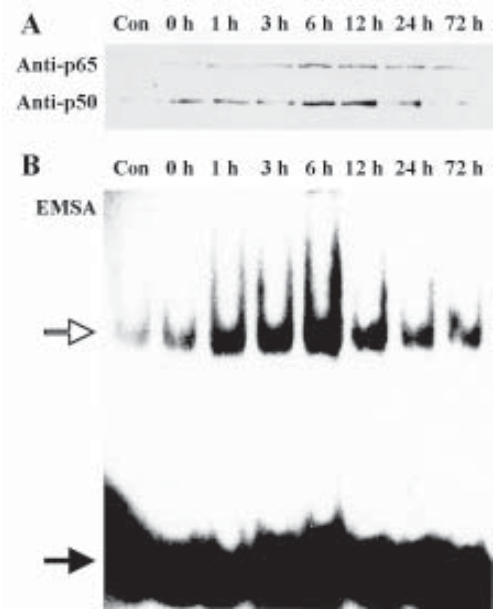

Fig 2. Time course of I/R induced alterations of NF-κB subunits p65 and p50 proteins and DNA binding activity from sham and ischemic rats that had 0, 1, 3, 6, 12, 24, and 72 h of reperfusion. A: Western blot analysis with anti-p65, p50 antibodies. B: EMSA of NF-κB DNA binding activity. The open arrowheads indicate the position of NF-κB DNA binding complexes. The filled arrowheads indicate the position of unbound DNA probes. *n*=4 independent animals.

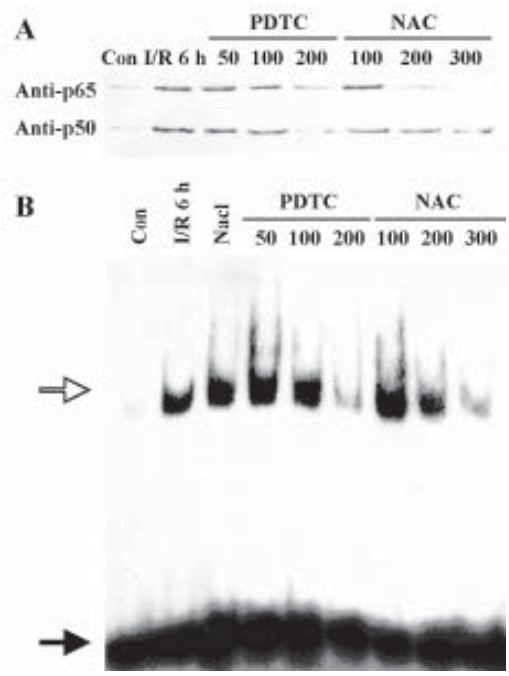

Fig 3. Effects of PDTC and NAC on I/R induced alterations of NF-κB subunits p65 and p50 proteins and DNA binding activity after I/R 6 h following 4-VO. A: Western blot analysis of hippocampal extracts with anti-p65 and anti-p50 antibodies derived from rats pretreatment with PDTC or NAC. B: EMSA of NF-κB DNA binding activity prepared from rats pretreatment with PDTC or NAC or with vehicle. The open arrowheads indicate the position of NF-κB DNA binding complexes. The filled arrowheads indicate the position of unbound DNA probes. C: Bands corresponding to p65 or p50 or binding bands were scanned and the intensities were represented as folds *vs* sham control (Tab 1, sham control=1, Mean±SD. $^{b}P<0.05$ *vs* sham control, $^{e}P<0.05$ *vs* I/R6 h+ PDTC100, $^{h}P<0.05$ *vs* I/R6 h+NAC100, $^{k}P<0.05$ *vs* I/R6 h+ NAC200. *n*=3 independent animals).

manner. NAC (200 mg/kg) reduced the NF-κB DNA binding activity by 60 % and pretreatment with NAC (300 mg/kg) resulted in a complete inhibition. Western blot analysis of homogenates from nuclear extracts in rat hippocampus was performed with anti-p65 and p50 antibodies. The trends of changes with Western blot were similar to with EMSA. The similar results were also obtained by intraperitoneal injection of PDTC (50-200 mg/kg). PDTC (200 mg/kg) showed a significant inhibition of NF-κB inductions and DNA binding activity (Fig 3).

Loss of IκBα protein in cytoplasm and effects of antioxidants on ischemia-induced loss of IκBα in rat hippocampus To evaluate the role of inhibitory protein IκBα in NF-κB activation, western blots were carried out to study the time course of IκBα protein in the cytoplasm after ischemia. It showed that IκBα and pIκBα peaked their levels at 3 h or 24 h respectively[5]. To evaluate the effects of antioxidants on the alterations of IκBα and pIκBα in the cytoplasm, PDTC or NAC

Tab 1. Pretreatment with PDTC or NAC on the alterations of p65, p50 protein express and NF-κB DNA binding activity.

Method	I/R 6 h	I/R 6 h+PDTC (mg/kg)			I/R 6 h+NAC (mg/kg)		
		50	100	200	100	200	300
Anti-p65	4.79±0.78^{b}	3.37±0.71	3.10±0.97^{e}	1.11±0.74^{e}	4.78±0.44	1.72±0.47^{h}	0.64±0.39^{k}
Anti-p50	5.50±0.33^{b}	4.87±0.85	3.25±0.53^{e}	1.38±0.98^{e}	5.61±0.87	3.87±1.58^{h}	1.89±0.87^{k}
EMSA	4.93±0.95^{b}	5.69±1.03	4.31±1.17	2.20±0.86^{e}	4.82±0.77	3.66±0.55^{h}	0.61±0.65^{k}

was intraperitoneally injected to the 4-VO rats 20 min before 30 min ischemia. The results suggested that PDTC (200 mg/kg) or NAC (300 mg/kg) significantly prevented the I/R-induced decrement of IκBα (from 0.50±0.10 to 0.80±0.20 or 1.20±0.24, respectively, sham control=1) and I/R-induced increment of pIκBα (from 2.00±0.15 to 0.46±0.10 or 0.41±0.10, respectively) from the cytoplasm of ischemic hippocampus (Fig 4).

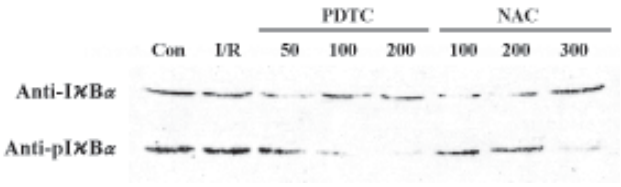

Fig 4. Effects of antioxidants on I/R induced alterations of IκBα and pIκBα proteins after I/R following 4-VO. Effects of PDTC and NAC on I/R induced alterations of IκBα and pIκBα PDTC or NAC of 50-300 mg/kg was administered to the rats 20 min before ischemia. Then hippocampi were removed at 3 h or 24 h reperfusion. *n*=3 independent animals.

DISCUSSION

Our previous studies demonstrated that NF-κB is continuously activated in nuclear extracts from hippocampus of rats following transient global brain ischemia[5]. Transient global brain ischemia caused a substantial up-regulation of p65 and p50 proteins which were seen as early as 0 h after ischemia, and their peak levels were observed after I/R 6 h. The NF-κB DNA binding activity is an approximate 7-fold after I/R 6 h when compared with the control rats. But there is no variation of p65 and p50 protein expression in cytoplasm (data not shown).

Many factors are known to activate NF-κB, such as ROS[13], calcium overload[14], and various cytokines[15], which have been implicated as causative agents in I/R insults. Our previous studies suggested that NF-κB was mediated by NMDA, non-NMDA receptor and L-VGCC channel non-selectively[5]. NF-κB may take a pro-apoptotic effect to neurons survival. In order to further get other evidence, PDTC, a commonly used scavenger of ROS, was used at 20 min before 30 min ischemia.

ROS can modulate the expression of immune and inflammatory genes and have an important role in leading to neuronal death. But the mechanism of the effect of antioxidants to cell death in postischemic brain is still unclear. To understand the relationship among ROS, NF-κB and ischemic injury, we need to develop an understanding of how ROS activate NF-κB and how NF-κB leads to cell death. We decided to examine the effect of PDTC, in order to provide the evidence that NF-κB activation is correlated with ROS after brain ischemia. We also used another antioxidant (NAC) as the reference to evaluate the effectiveness of NF-κB to cell death. Our studies suggest that pretreatment with PDTC or NAC attenuates the NF-κB induction and activation. Furthermore, both of them improve the cell survival of the hippocampal CA1 pyramidal cells through histological observation. These data show that PDTC and NAC can strongly protect neurons from I/R injury and suggest that NF-κB is involved in ROS-induced injury. The ability of these two antioxidants to inhibit NF-κB activation suggests that ROS may be a second messenger system for activation of NF-κB. Moreover, some antioxidants, such as melatonin[16], have been proposed and used as potential therapeutic agents against oxidative stress-induced neuronal cell death, so NF-κB may be a novel therapeutic target for global brain ischemia.

It is known that NF-κB is trapped in cytoplasm by IκBα. When cells are stimulated, NF-κB is activiated and then translocated into the nucleus by phosphorylation and degradation of IκBα[12]. Then, NF-κB binds to a κB-specific DNA motif and regulates transcription of genes. In our previous studies, degradation and subsequent re-synthesis of IκBα have been observed after I/R different times[5,6]. Here we also found that ischemia-induced IκBα degradation and pIκBα increment in the cytoplasm as well as NF-κB activation in the nucleus could be blocked by PDTC and NAC. It suggests that IκBα degradation is followed by phosphorylation of IκBα, and IκBα controls NF-κB activation while active NF-κB promotes IκBα expression in turn. ROS may lead to the phosphorylation of IκBα. The newly synthesized IκBα protein replenishes the cytoplasm to re-establish inactive NF-κB complexes[17].

In conclusion, antioxidants of PDTC and NAC can inhibit the inductions of p65, p50 subunits and NF-κB DNA binding activity in the nucleus, which also prevent the loss of IκBα in the cytoplasm. The protective effects of antioxidants against ischemic-induced injury may be mediated by down-regulation of NF-κB activation.

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