Modulation of IκB kinase autophosphorylation and activity following brain ischemia[1]

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ABSTRACT

AIM: To investigate the effects of different antagonists on the alteration of IκB kinase (IKK) activity in rat hippocampus following global brain ischemia. **METHODS:** Using 4-vessel occlusion (4-VO) as brain ischemia model, IKK protein expression was examined by immunoblotting and immunoprecipitation, and IKK activity was assayed by *in vitro* kinase assay. **RESULTS:** There was no alteration of IKK protein expression following ischemia or ischemia/reperfusion different time points, but IKK activity reached its peak level at ischemia 30 min. Pretreatment with *N*-methyl-*D*-aspartate (NMDA) receptor antagonist ketamine, non-NMDA receptor antagonist DNQX, or NF-κB inhibitor PDTC decreased the IKK activity following brain ischemia 30 min. The increase in substrate myelin basic protein (MBP) phosphorylation by IKK is associated with an increase in autophosphorylation of IKK, which can also be antagonized by ketamine, DNQX, and PDTC. **CONCLUSION:** NMDA receptor and non-NMDA receptor mediate the increase of IKK activity following global brain ischemia in rat hippocampus, which contributes to the alterations of expression and activity of downstream factor NF-κB.

INTRODUCTION

We have previously shown that the increased nuclear basal NF-κB expression (p65 and p50) and activity result from an increased cytoplasmic IκB-degradation, and are mediated by *N*-methyl-*D*-aspartate (NMDA) and non-NMDA receptor and L-type voltage-gated Ca^{2+} channel following severe global ischemia in rat hippocampus[1]. Some studies revealed that both NF-κB-inducing kinase (NIK)[2] and mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1)[3,4] can activate a multiprotein (IKK) complex of M_r 700 000-900 000, which is responsible for directly phosphorylating I-κB proteins. Several pathways of NF-κB activity are thought to converge at the level of IκB kinase (IKK) activation, implicating this complex as a critical regulator of NF-κB transcriptional regulation. The IKK complex consists of 2 catalytic components, IKKα and IKKβ, as well as a regulatory IKKγ subunit. Phosphorylation of serine residues (at positions 176 and 180 in IKKα or positions 177 and 181 in IKKβ) by upstream kinases, including NIK and MEKK1, activates IKK kinase. Mutation of these serine residues to alanine leads to prolonged IKK activation, suggesting that phosphorylation of these residues, likely by autophosphorylation, functions to down-regulate IKKβ activity[5]. The activation of the IKK complex could be achieved through IKKγ-mediated oligomerization, indicating that IKKγ

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functions as an adaptor to link the IKKs with NF-κB[6]. Biochemical analysis demonstrated that both IKKα and IKKβ kinases phosphorylated IκBα at serines 32 and 36 or IκBβ at serines 19 and 23. Thus, both IKKα and IKKβ contribute to the activity of the IKK complex and are involved in NF-κB activation.

Recent studies have found that the mechanisms of ischemic neuronal death have been focused on glutamate receptor activation and subsequent elevation of intracellular Ca^{2+} concentration. They also suggested that stimulation of glutamate receptors strongly activates NF-κB *in vitro* and is implicated in the pathogenesis of cerebral ischemia and other neurodegenerative disorders[7,8]. But how NMDA or non-NMDA receptor mediates NF-κB expression and activation is to be determined. In this study, we detect the IKK activity by *in vitro* kinase assay to illuminate the mechanism of the regulation of IKK by NMDA or non-NMDA receptor after rats were subjected to 30-min 4-vessel occlusion (4-VO).

MATERIALS AND METHODS

Materials Rabbit polyclonal IKKα/β (sc-7607) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Alkalline phosphatase conjugated goat anti-rabbit IgG was from Sigma (St Louis, MO, USA). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Promega (Madison, WI, USA). MBP was from Sigma (M1891, USA). [γ-^{32}P] ATP was from Yahui Biological and Medical Engineering Co, Beijing. Ketamine, nifedipine, 6,7-dinitroquinoxaline-2,3-(1*H*,4*H*)-dione (DNQX), pyrrolidine dithiocarbamate (PDTC), and *N*-acetylcysteine (NAC) were all from Sigma.

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 four-vessel occlusion, as described before 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 following day, ischemia was induced by occluding bilateral vertebral arteries with aneurysm clips for 5, 10, or 30 min, respectively. During the ischemia, animals were required to match the following criteria: completely flat electroencephalogram (EEG), maintenance of dilated pupils, absence of a cornea reflex after exposure to strong light stimulation, and maintenance of rectal temperature at about 37 ° C. Animals not matching these criteria or with seizures were all excluded. Control animals received the same surgical procedures except bilateral carotid arteries were not occluded.

Brain tissues and drug treatment Cytosolic proteins were extracted by a modification of a previously described procedure[10]. Briefly, tissue samples were homogenized in ice-cold buffer A [HEPES 10, $MgCl_2$ 0.5, KCl 10, ethylene diamine tetraacetic acid (EDTA) 0.1, glycol-bis-(α-aminoethyl) ether *N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) 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 of 10 % NP-40, the homogenates were vortexed for 30 s and then centrifuged at 800×*g* for 15 min. Supernatants were aliquoted and frozen in liquid nitrogen, and stored at -80 ° C until use.

Cytosolic extracts were used for IKK expression by immunoblotting analysis and IKK activity by *in vitro* kinase assay. To evaluate the effects of different antagonists on ischemia-induced alterations of IKK, animals were given ketamine (50 mg/kg, ip), DNQX (30 mg/kg, ip), nifedipine (20 mg/kg, ip), PDTC (200 mg/kg, ip), and NAC (300 mg/kg, ip), respectively 20 min before 30 min of ischemia. Control rats were received ip of vehicle (0.9 % NaCl or Me_2SO).

Western blot Western blot analysis was performed as described previously[11] with some modifications. Protein concentrations were determined by Lowry method[7]. Samples were mixed with loading buffer and boiled for 5 min. Proteins were separated on 10 % SDS-PAGE gel using constant voltage, then were subsequently electroblotted onto NC membranes (Millipore, Bedford, MA) with a semidry blotting system. After blockade in PBS with 0.1 % Tween-20 (PBST) and 3 % bovine serum albumin (BSA) for 2 h, membranes were incubated overnight at 4 ° C with primary antibodies in PBST containing 3 % BSA. Membranes were then washed and incubated with an alkaline phosphotase-conjugated secondary antibody for 2 h. Immunoreactivity was detected by NBT/BCIP assay kit.

Immunoprecipitation and *in vitro* kinase assay A 500 μg sample of the lysate was used for

immunoprecipitation. The cytosolic lysate was incubated overnight at 4 °C with IKKα/β antibody. In the next day the 20 μL agarose-conjugated beads were added to the tube and incubated at 4 °C for 2 h. The immunoprecipitates were washed extensively with immunoprecipitation buffer thrice and once with kinase buffer (Tris-HCl 20, $MgCl_2$ 10, DTT 0.5, PMSF 1, ATP 0.01 mmol/L; pH 7.6). *In vitro*, the kinase assay was performed with MBP protein in 20 μL of kinase buffer containing 74 kBq of [γ-^{32}P] ATP and 5 μg of the substrate MBP at 30 °C for 30 min. Samples were added 5 μL of 5×SDS loading dye to each reaction, boiled for 5 min, analyzed by 12.5 % SDS-PAGE and autoradiography using Kodak X-Omat film.

Statistical analysis Western blot and autoradiographic results were semiquantitatively evaluated by means of an image analyzer (Lab Works Software, UVP upland, CA). Values were expressed as mean±SD from 3 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 $P<0.05$ was considered significant.

RESULTS

Time course of ischemia-induced activation of IKK in rat hippocampus after ischemia and ischemia/reperfusion To evaluate the alteration of IKK protein expression and activation, rats were sacrificed at ischemia 5, 15, 30 min or after ischemia/reperfusion (I/R) 1, 6 h. As shown in Fig 1A, B, the results of immunoprecipitation and Western blot clearly show two bands, which represent the two subunits of IKKα and IKKβ (M_r 85 000 and 87 000, respectively). There was no significant alteration of IKKα or IKKβ protein following ischemia different time points or I/R different time points. *In vitro* kinase assay of homogenates from cytoslic extracts in rat hippocampus was immunoprecepitated with IKKα/β antibody and detected with Ser/Thr phosphorylated substrate of myelin basic protein (MBP). The results of *in vitro* kinase assay indicated that the alteration of either autophosphorylation of IKK or substrate phosphorylation of MBP was significantly different (Fig 1C, D). The substrate phosphorylation of MBP increased with the extent time of ischemia, reached its peak level at ischemia 30 min, and decreased with the extent time of I/R. The variation rule of IKK autophosphorylation *in vitro* was similar to

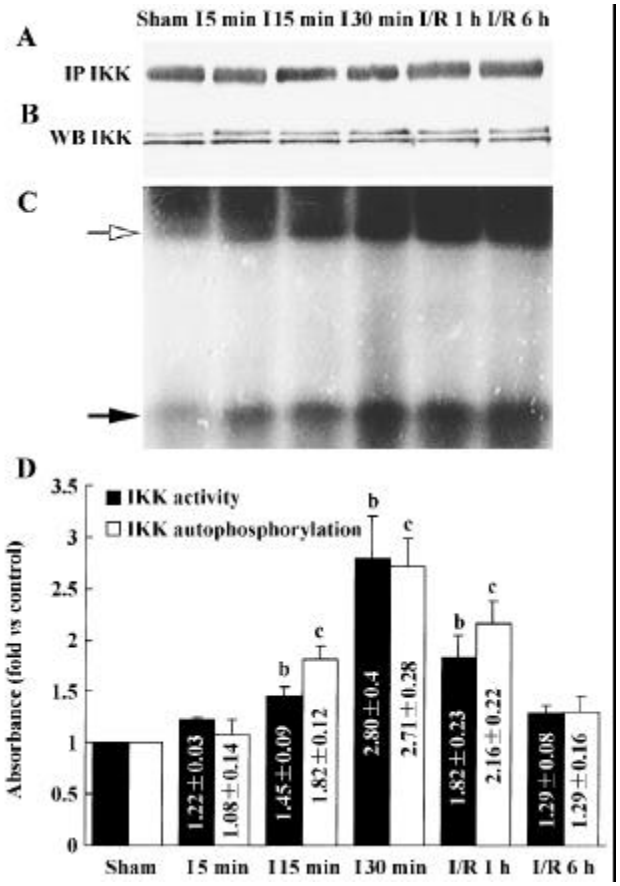

Fig 1. Time course of ischemia- or ischemia/reperfusion (I/R)-induced alterations of IKK activity from sham and ischemic rats that had 5-, 15-, 30-min ischemia and I/R rats that had 1-, 6-h reperfusion. A: Immunoprecipitation (IP) analysis with anti-IKKa/b antibody showing two bands of M_r 85 000 and 87 000. B: Western blot (WB) analysis with anti-IKKa/b antibody showing two bands of M_r 85 000 and 87 000. C: *In vitro* IKK kinase assay with Ser/Thr phosphorylated substrate MBP. D: Bands corresponding to IKK protein expression or IKK activity were scanned and the absorbance was represented as fold *vs* sham control. n=3 independent animals. Mean±SD. $^bP<0.05$ *vs* IKK activity in sham control. $^cP<0.05$ *vs* IKK autophosphorylation in sham control. The open arrowhead indicates the position of IKK autophosporylation. The filled arrowhead indicates the position of phosphorylated substrate MBP.

the alteration of IKK activity except that the beginning time of autophosphorylation increment preceded that of the increase of IKK activity.

Effects of antagonists ketamine, DNQX, and PDTC against ischemia-induced activation of IKK Our previous studies have shown that PDTC, an NF-κB inhibitor, can decrease the expression of NF-κB subunit (p65 and p50) and DNA binding activation of NF-κB following I/R 6 h (data not shown). To further

illuminate the relationship between upstream kinase of IKK and downstream effecter of NF-κB, PDTC was administered 20 min before 30-min ischemia. PDTC (200 mg/kg) showed a significant inhibition of IKK activaity (Fig 2). To verify whether activation of IKK after ischemia was associated with NMDA receptor, non-NMDA receptor, or L-type voltage-gated Ca^{2+} channel (L-VGCC), we intraperitoneally injected 4-VO rats with three antagonists ketamine, DNQX, and nifedipine. The ischemia-induced increase of IKK activity in rat hippocampus of cytosolic extracts was significantly inhibited by pretreatment with ketamine (50 mg/kg) and DNQX (30 mg/kg) but not by nifedipine (20 mg/kg) or NAC (300 mg/kg, Fig 2). These results indicated that IKK activity might be mediated by NMDA receptor and non-NMDA receptor, but not by L-VGCC.

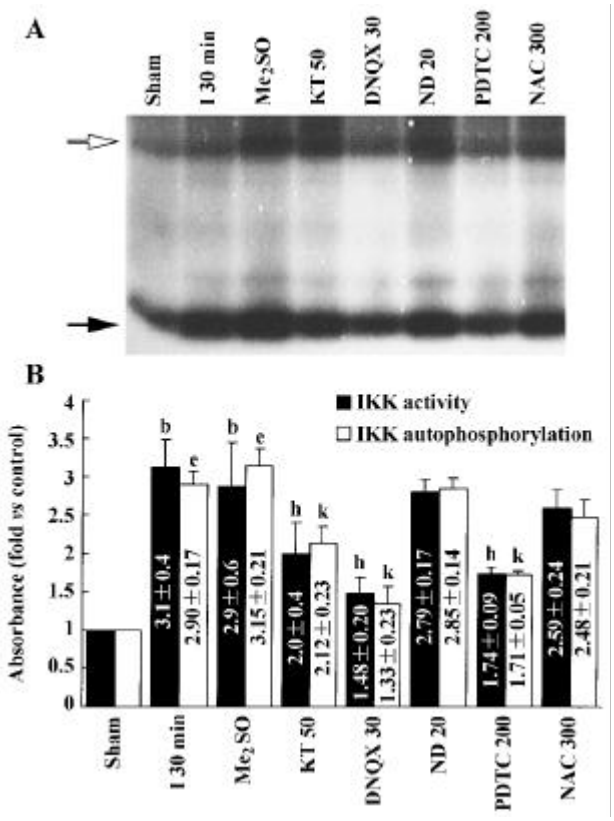

Fig 2. Effects of KT, DNQX, and PDTC on I/R-induced alterations of IKK activity after ischemia 30 min following 4-VO. A: *In vitro* IKK kinase assay of cytoplasmic extracts from hippocampus with anti-IKKa/b antibody derived from rats subjected to 4-VO, treated with KT (50 mg/kg), DNQX (30 mg/kg), ND (20 mg/kg), PDTC (200 mg/kg) or NAC (300 mg/kg) 20 min before 30-min ischemia. B: Bands corresponding to IKK activity were scanned and the absorbance was represented as fold *vs* sham control. *n*=3 independent animals. Mean±SD. $^{b}P<0.05$ *vs* IKK activity in sham control. $^{e}P<0.05$ *vs* IKK autophosphorylation in sham control. $^{h}P<0.05$ *vs* IKK activity in Me_2SO. $^{k}P<0.05$ *vs* IKK auto-phosphorylation in Me_2SO. The open arrowhead indicates the position of IKK autophosphorylation. The filled arrowhead indicates the position of phosphorylated substrate MBP.

DISCUSSION

IKKα and IKKβ may normally exist as a leucine zipper-linked heterodimer that can interact directly with the upstream kinase NIK[12], which are similar in structure and thought to have similar function-phosphorylation of the IκB inhibitors in response to stimuli. Thus, we use IKKα/β as the exact antibody to evaluate the expression and activity of both IKKα and IKKβ.

In the present study, we examined the expression, activity, and autophosphorylation of IKK complex following global brain ischemia in rat hippocampus. We assayed IKK kinase activity *in vitro* in each fraction using MBP as a Ser/Thr phosphorylating substrate. Kinase specificity was established by replacing IKK antibody or MBP substrate with one another and keeping all other procedures unchanged. Results of Western blot or immunoprecipitation showed that the IKK protein level remains the same without changes. But we observed that IKK activity increased with the extent time of ischemia and reached its peak level at ischemia 30 min and decreased with the time of I/R by *in vitro* IKK kinase assay. And the autophosphorylation of IKK *in vitro*, locating in the site of IKK protein bands, showed a similar change with the alteration of IKK activity. Taken together, it suggested that the alterations of IKK activity and autophosphorylation might not be contributed to IKK protein expression. Furthermore, there was a constitutive level of IKK activity in sham control rats, indicating that a basal level of IKK activity might be required to maintain normal cell functions, such as cell survival or differentiation.

We have previously demonstrated that NMDA receptor antagonist ketamine, non-NMDA receptor antagonist DNQX, and L-VGCC antagonist nifedipine markedly inhibit the NF-κB expression and activity[1]. This study showed that NMDA receptor, non-NMDA receptor, and antioxidant PDTC decreased the activity of IKK complex. It suggested that brain ischemia-induced activation of IKK led to increased phosphorylation and degradation of IκBα and released active NF-κB, which translocates to nucleus and transcriptionally activates responsive genes. In addition, newly synthesized IκBα enters the nucleus and prevents NF-κB-mediated transcription of target genes. These results

and our previous studies indicate that IKK complex is a common upstream kinase for NF-κB activation through members of the TNFR superfamily and their signal transducers, TRAFs and NIK following brain ischemia[13]. NF-κB is a signal transducer through upstream kinase IKK, which transmits glutamate and Ca^{2+} signals from distant sites to nucleus and IKK activity is mediated by NMDA and non-NMDA receptors.

Reactive oxygen species (ROS) can modulate the expression of immune and inflammatory genes and have an important role in leading to neuronal death. NF-κB was the first eurkaryotic transcription factor shown to respond directly to ROS[14]. To further investigate the interaction of ROS with IKK and NF-κB, we examined the two important antioxidants of PDTC (also NF-κB inhibitor) and NAC following 30-min ischemia. Our studies showed that IKK activity was significantly inhibited by PDTC but not by NAC. It indicated that the inhibitory function of PDTC might be through inhibiting IKK activity, while NAC might inhibit NF-κB activity and expression directly through functioning on inhibitory protein IκBs.

As known to all, IKK *in vitro* kinase assay showed a back-phosphorylation, which means that the higher autophosphorylation *in vitro* is equivalent to the lower autophosphorylation *in vivo*. In this paper, we observed that the autophosporylation of IKK preceded the activation of IKK. The increase in substrate MBP phosphorylation by IKK is associated with an increase in autophosphorylation of IKK[15] and can be antagonized by ketamine, DNQX, or PDTC. It suggested that activation of IKK was followed by the IKK autophosphorylation, then the activated IKK phosphorylated the substrate of MBP *in vitro*. The increase and decrease of autophosphorylation lead to variation of IKK activity, suggesting that autophosphorylation functions to regulate IKK activity[5].

In conclusion, IKK autophosphorylation and activity are mediated by NMDA and non-NMDA receptor. Blocking the IKK-NF-κB signal transduction pathway may have therapeutic potential for the treatment of ischemia injury.

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