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Calcium Signal-Initiated Early Activation of NF-κB in Neurons Is a Neuroprotective Event in Response to Kainic Acid-Induced Excitotoxicity

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Abstract—We demonstrate that activation of nuclear factor κB (NF-κB) in neurons is neuroprotective in response to kainic acid (KA)-induced excitotoxicity. Combination of Western blotting, immunocytochemistry, and electrophoresis mobility shift assay showed that KA exposure induced a fast but transient nuclear translocation of the NF-κB p65 subunit and increased DNA-binding activity of NF-κB in primary cultured cortical neurons. The transient NF-κB activity was associated with upregulation of antiapoptotic Bcl-xL and XIAP gene products revealed by real-time PCR. Knockdown of p65 decreased neuronal viability and antiapoptotic gene expression. In addition, we showed that KA-stimulated DNA-binding activity of NF-κB was associated with reactive oxygen species and calcium signals, using AMPA/KA receptor antagonist, calcium chelator, and antioxidant. These results suggest that the fast and transient activation of NF-κB initiated by calcium signals is one of the important proximal events in response to KA-induced excitotoxicity, which has neuroprotective effect against KA-induced apoptosis.

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Key words: NF-κB, kainic acid, ROS, calcium, AMPA/KA receptor, neuronal survival/death

Central nervous system neurons bearing glutamate receptors are vulnerable to excitotoxicity, a feature whereby excessive activation of glutamate receptors triggers cell death [1]. Kainic acid (KA) is a non-degradable analog of glutamate that is more potent than glutamate as a neuronal excitant. KA induces selective degeneration of neurons, especially in striatal and hippocampal areas of the brain after intraventricular and intracerebral injections [2].

Nuclear factor (NF)-κB was originally identified as a transcription factor binding to the enhancer of the kappa immunoglobulin light chain in B cells [3]. Transcription factors of the NF-κB family are now regarded as ubiquitous factors responsible for activating genes crucial for a series of cellular processes such as cell survival, apoptosis, development, oncogenesis, and immune and inflammatory responses [4-6]. NF-κB is a homo- or heterodimeric protein of NF-κB/Rel family members (RelA/p65, RelB, c-Rel, p50, and p52) in mammalian cells [4]. The p50/p65 dimer is the most common form of NF-κB factors. In unstimulated cells, NF-κB dimers are normally inactivated in the cytosol by interaction with the inhibitory IκB proteins that mask their nuclear localization signal [4, 7, 8]. Activation of NF-κB by cytokines or B-cell activating factor is triggered via a classical or alternative pathway in which inhibitory IκB protein is degraded in a NEMO (also known as IKKγ)-dependent or -independent way, and then NF-κB is released and translocated to the nucleus where it activates target genes [4-6, 9]. In addition, NF-κB can be activated by UV radiation [10] or

Abbreviations: AMPA, α-aminomethylisoxazole-propionic acid; CTZ, cyclothiazide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; KA, kainic acid; MTT, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; PMSF, phenylmethylsulfonyl fluoride; RNAi, RNA interference; ROS, reactive oxygen species.

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oxidative stress [9, 11] via other nonconventional pathways.

NF-κB is highly expressed in both the vertebrate and invertebrate central and peripheral nervous systems. In the central nervous system, NF-κB is modulated under physiological and pathological conditions. Physiologically, NF-κB functions during neural development and synaptic plasticity [12, 13]. Under pathological conditions, NF-κB is thought to regulate processes related to disease and injury. For instance, NF-κB is implicated in the cellular response to brain injury, seizure, and neurodegenerative diseases such as Alzheimer's and Parkinson's [12, 13]. In models of excitotoxicity and seizures, glutamate or AMPA (α-aminomethylisoxazolepropionic acid)/KA elicits a rise in intracellular free Ca^{2+}, triggering mitochondrial ROS (reactive oxygen species) production [1, 2, 14]. ROS can activate NF-κB [9, 11]. Thus, NF-κB activity is greatly increased in brain cells following excitotoxic and apoptotic insults. However, the functions of NF-κB in the nervous system are controversial. Several studies have demonstrated that induction of NF-κB activity stimulates antiapoptotic proteins, thereby being neuroprotective [15-19]. However, others report that NF-κB activation leads to neuronal death [20-22].

In this study, we demonstrate that KA can trigger a faster but more transiently nuclear translocation of the NF-κB p65 subunit at the earlier stages of KA exposure, which is initiated by calcium signals. The transient NF-κB activity is associated with upregulation of some antiapoptotic gene products such as Bcl-xL and XIAP, thereby protecting against KA-induced neuronal death. Our data suggest that early activation of NF-κB is an important event in response to KA-induced excitotoxicity.

MATERIALS AND METHODS

Chemicals and materials. Kainic acid (KA), NBQX, D-AP5, and cyclothiazide (CTZ) were from Tocris Cookson (UK). BAPTA-AM and vitamin E were obtained from Sigma (USA). Fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), and B27 were from Invitrogen (USA). Culture dishes and plates were purchased from Corning Glass (USA). Other reagents including poly-L-lysine were purchased from Sigma.

Primary culture and KA exposure. Primary cerebral cortical neurons were cultured as described previously [23] with modification. Cerebral cortices were isolated from the brains of newborn (P0) Sprague–Dawley rats. The cortices were immediately immersed in 1× D-Hanks solution supplemented with 5 mM glucose. Meninge-stripped brains were minced and digested at 37°C for 15 min in 0.25% trypsin. After centrifugation of digests, the pellet was dissociated and the suspension was filtered though a 70-μm nylon mesh. The cells were counted and plated at a density of $1 \cdot 10^6$ cells/cm^2 in DMEM with 20% calf serum onto 24-well dishes or 100-mm dishes precoated with 1% poly-L-lysine and grown in a 37°C incubator with 5% CO_2. After cells attached to the plates, the culture medium was replaced with neurobasal medium supplemented with 2% B27, 2 mM glutamine. Cortical neurons were cultured for 7 days (DIV7) before KA exposure.

Cell viability and toxicity assays. Cell toxicity and viability assays were performed as described previously [24]. After treatments with KA for given times, neurons were assayed for viability using 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT; Sigma), which was added at a final concentration of 1 mg/ml for 4 h. MTT was removed, and neurons were lysed in 200 μl of dimethyl sulfoxide. Absorbance was measured at 570 nm on a microplate reader (Bio-Rad model 680; Bio-Rad, USA). Each condition was tested in four to six wells, and at least three independent experiments were performed. The data are expressed as a percentage of untreated neurons that remained in the presence of KA.

Nuclear extract preparation and electrophoresis mobility shift assay (EMSA). Neurons ($1 \cdot 10^6$) were collected by centrifugation at 800*g* for 5 min. Nuclear extracts were prepared as described previously [23]. Neurons were lysed in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.6% NP-40) and centrifuged at 1500*g* for 15 min at 4°C. The supernatant was used as cytosolic protein. Pellets were lysed in 15 ml of high-salt buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) for 20 min on ice. Storage buffer (75 ml; 20 mM Hepes, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) was added. The resulting nuclear pellets were resuspended by vortex mixing and then centrifuged at 6000*g* for 20 min. The resulting supernatant was used as soluble nuclear proteins. For Western blotting, samples (30 μg protein per lane) were probed with anti-NF-κB p65 (Santa Cruz, USA). EMSA was performed as described previously [23]. Binding reactions were performed at room temperature for 10 min using 2 μg of nuclear protein with a biotin-labeled probe, followed by resolution of the DNA–protein complexes on native 4% polyacrylamide gels. Double-stranded oligodeoxynucleotides containing a NF-κB-binding consensus sequence (probe) were as follows (only the upper strand is indicated): NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; NF-κB-mutant, 5′-AGTTGAGGCGACTTTCCCAGGC-3′. In competition experiments, 200-fold molar excess of unlabeled oligonucleotides was used. Signals were detected by chemiluminescent imaging according to the manufacturer's protocol (EMSA Gel-Shift Kit; BioEol, PR China).

Fluo-3 fluorescence measurements. Cortical neurons plated on specific dishes were loaded by incubation with

5 μM fluo-3 AM for 30 min at 37°C in PSS buffer (127 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM glucose, 10 mM Hepes, pH 7.4). After incubation, the dishes were washed and placed on the stage of a laser confocal microscope (Leica TCS SP2; Leica Microsystems Heidelberg GmbH, Germany). Ca^{2+} imaging experiments were dynamically performed under the laser confocal microscope. Cortical neurons were excited at 480 nm, and the emitted fluorescence was passed through a 530 nm bandpass filter and collected with a 20× objective. The neurons were recorded for at least 30 sec before the agonist stimulation and thereafter fluorescence changes were recorded for 300 sec. Image analysis was carried out with the laser confocal microscope-assisted multiple excitation magical imaging system. Areas of the cell bodies were drawn and the averaged value of pixel intensities was evaluated at a running time point.

Western blotting. Western blotting was performed as described [24]. Thirty micrograms of total protein was separated in 12% polyacrylamide gel and transferred onto nitrocellulose. The membranes were blocked and probed with specific antibodies against procaspase-3 (1 : 1000; Cell Signaling, USA) or β-actin (1 : 500; Santa Cruz). After washing, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies. Chemiluminescence signals were visualized using Western blotting luminol reagent (Santa Cruz).

Immunostaining. This experiment was performed as described [25] with modifications. On day 8 in culture, cortical neurons were treated with 100 μM KA for 4 h. After two washing steps with neurobasal medium, cells were fixed for 10 min in 3.7% formaldehyde and then permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 20 min at room temperature. After washing with PBS, cells were incubated in 10% goat serum for 30 min at room temperature, followed by two 5-min washes with PBS. Cells were then incubated with anti-p65 polyclonal antibody (1 : 50 dilution; Santa Cruz) at 37°C for 2 h, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (1 : 100 dilution; Santa Cruz) at room temperature for 1 h. Nuclei were stained with Hoechst 33342, and images were acquired with the laser confocal microscope.

Real-time quantitative PCR. Total RNAs were extracted using TRIzol reagent (Invitrogen, USA). Two micrograms of RNAs were subjected to reverse transcription by M-MLV reverse transcriptase (Promega, USA). PCR amplification was performed in triplicate using the following primers: for XIAP, (sense) 5′-GGGGAATCTGTGGTAAGAAC-3′ and (antisense) 5′-ACTTGACTCATCCTGCGAAT-3′; for Bcl-xL, (sense) 5′-TGAATGACCACCTAGAGCCT-3′ and (antisense) 5′-GAGCCCAGCAGAACTACACC-3′; for β-actin, (sense) 5′-GAAGTGTGACGTTGACAT-3′ and (antisense) 5′-ACATCTGCTGGAAGGTG-3′. Triplicate 25-μl real-time PCR reactions were run, containing 12.5 μl SYBR Green PCR master mix, 1 μl of a primer stock solution containing 10 μM of both forward and reverse primers, 1 μl of cDNA template, and 10.5 μl nuclease-free water. Real-time PCR was performed using an ABI PRISM1 7300 Real-Time PCR system (Applied Biosystems, USA). The thermal profile for the real-time PCR was 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 57°C for 20 sec. Melting curve analysis was performed to measure the specificity of PCR product. The quantity of Bcl-xL or XIAP mRNA (C_t of Bcl-xL or XIAP) was normalized by subtracting the quantity of β-actin mRNA (C_t of β-actin) (internal control) to obtain a normalized value of $\Delta C_{t\text{-Bcl-xL}}$ or $\Delta C_{t\text{-XIAP}}$. A $\Delta\Delta C_t$ value was deduced by subtracting the exposed ΔC_t by the unexposed ΔC_t. The relative quantity of Bcl-xL or XIAP mRNA was obtained using the value of $2^{-\Delta\Delta Ct}$, according to the manufacturer's protocol.

Vector-based RNA interference (RNAi). A plasmid for RNA interfering was constructed by inserting a synthesized 64-bp oligonucleotide, containing a specific sequence targeting p65 mRNA, into the pSuper-basic vector (a kind gift from Yong-Feng Shang, Peking University Health Science Center) to generate pSuper-p65. The sequences for silencing p65 were synthesized as described previously [26]: 5′-GATCCCCGCAGTTCGATGCTGATGAATTCAAGAGATTCATCAGCATCGAACTGCTTTTTA-3′ (forward) and 5′-AGCTTAAAAAGCAGTTCGATGCTGATGAATTCAAGAGATTCATCAGCATCGAACTGCGGG-3′ (reverse). The oligonucleotides were resuspended in annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, 2 mM acetate) and heated to 95°C for 4 min, 70°C for 10 min, and then cooled to room temperature to generate double-stranded DNA. The double-stranded DNA was phosphorylated and subcloned into the *Bgl*II/*Hin*dIII-digested pSuper-basic vector. The vector was then transfected into cells with Lipofectamine 2000 reagent (Invitrogen). Transfection efficiency was monitored by cotransfection with a pEGFP construct and visualized with the laser confocal microscope.

Data analyses. Data are expressed as mean ± SD. Multiple comparisons were examined for significant differences using a one-way analysis of variance (ANOVA) followed by individual comparisons with the Tukey post-test. Statistical significance was set at $p < 0.05$.

RESULTS

Kainic acid-induced apoptosis in primary cultured cortical neurons. To evaluate the effects of glutamate receptor activation on cell death, we exposed neurons to KA for stimulating AMPA/KA receptors. Excitotoxic conditions were induced in cultured cortical neurons with various concentrations of KA (0, 25, 50, 100, 150 μM).

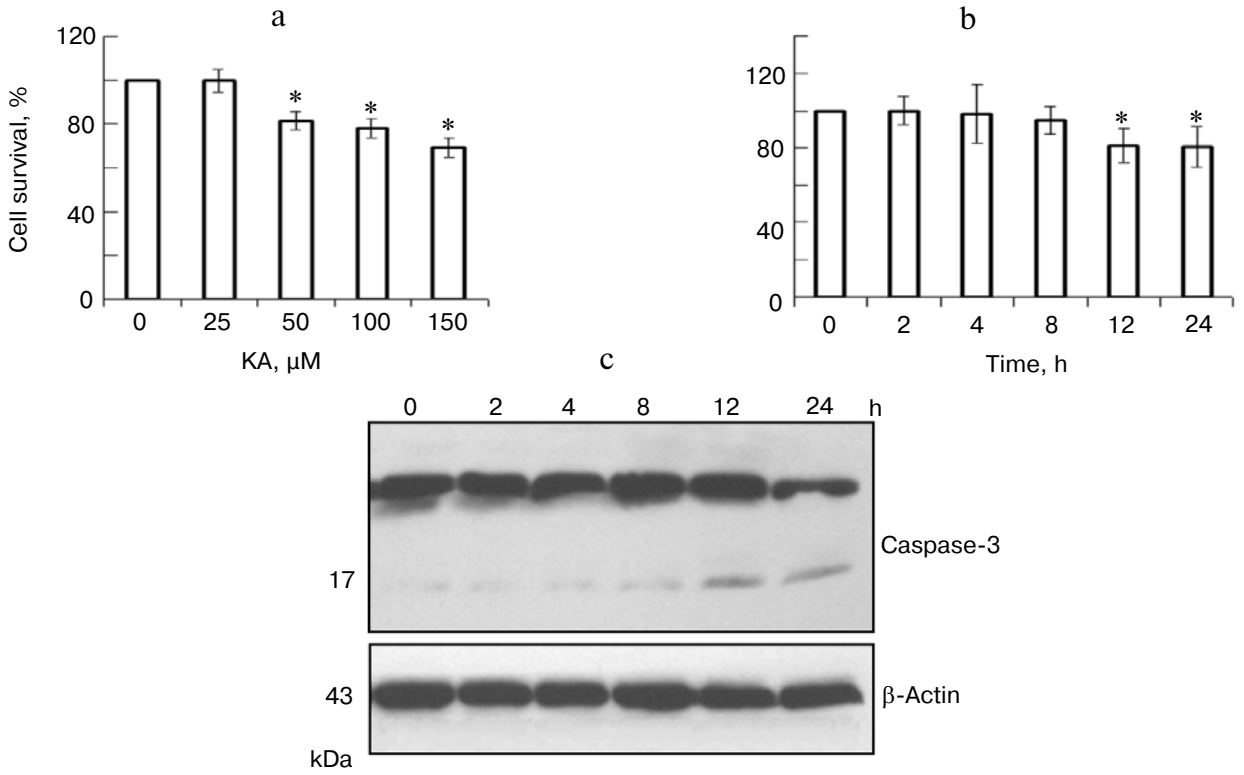

Fig. 1. KA-induced neuronal apoptosis in dose- (a) and time-dependent (b) manner: a) time of primary cortical neuron incubation with KA, 24 h; b) KA, 100 μM. Here and in Figs. 3b, 4b, 4c, and 5c, data represent mean ± S.D. derived from three separate cultures. Statistically significant differences of $p < 0.05$ are indicated by asterisks. c) Western blotting analysis of the activation of procaspase-3 in 100 μM KA-exposed neurons. β-Actin was used as a loading control.

After exposure for 24 h, a dose-dependent decrease in cell survival was revealed by the MTT method. Exposure to 50-150 μM KA decreased cell survival by >20% ($p < 0.05$) (Fig. 1a). Exposure of cortical neurons to 100 μM KA for 24 h elicited a time-dependent decrease in cell survival (Fig. 1b). To address whether this neuronal loss was attributed to apoptotic cell death, we examined the activation of procaspase-3. Procaspase-3 exists as a 32-kDa proenzyme that is proteolytically cleaved into 17-20 and 12 kDa subunits during activation [25]. Western blotting showed that the active 17-kDa subunit was detected within 12-24 h (Fig. 1c). These results indicate that KA-induced neuronal cell death is mediated by the caspase-dependent apoptotic pathway.

Induction of a fast and transient nuclear translocation of p65 by kainic acid. To investigate the role of NF-κB in response to KA-induced excitotoxicity, we first tested the levels of the p65 subunit of NF-κB after KA exposure using specific antibody. Western blotting showed that the levels of p65 protein in the nucleus increased immediately within 2-4 h after stimulation, then decreased but were still higher than that of control (0 h) within 8-12 h, thereafter its levels dropped to the basal level. The total amount of p65 was not changed in the cells (Fig. 2a; see color insert). These results indicate that KA exposure can trigger a faster but more transient nuclear translocation of the p50/p65 dimer at the earlier stages of exposure. This inference was further indicated by immunocytochemical staining and laser confocal microscopy, which showed that after KA exposure p65 was distributed in the nucleus as well as the neurites, while it was mainly localized in the cytosol and long neurites before exposure (Fig. 2b; see color insert). These results suggest that KA stimulation can trigger NF-κB activation at the early stages in response to KA-induced excitotoxicity.

Stimulation of NF-κB DNA-binding activity and anti-apoptotic gene expression by kainic acid. To identify whether the early and transient nuclear translocation of p65 played a role in the regulation of gene expression, the DNA-binding ability of NF-κB was analyzed by EMSA. When a biotin-labeled oligonucleotide (probe) containing a NF-κB binding consensus sequence was incubated with the nuclear extracts from the neurons exposed to KA for 4 h, an increase in DNA–nucleoprotein complex occurred (Fig. 3a). Although the DNA binding activities could be seen in unexposed and 24-h-exposed neurons, they were much weaker than that in 4-h-exposed neurons. To ascertain the specific NF-κB binding shift, we performed competition using unlabeled oligonucleotides containing NF-κB consensus or its mutant. The NF-κB

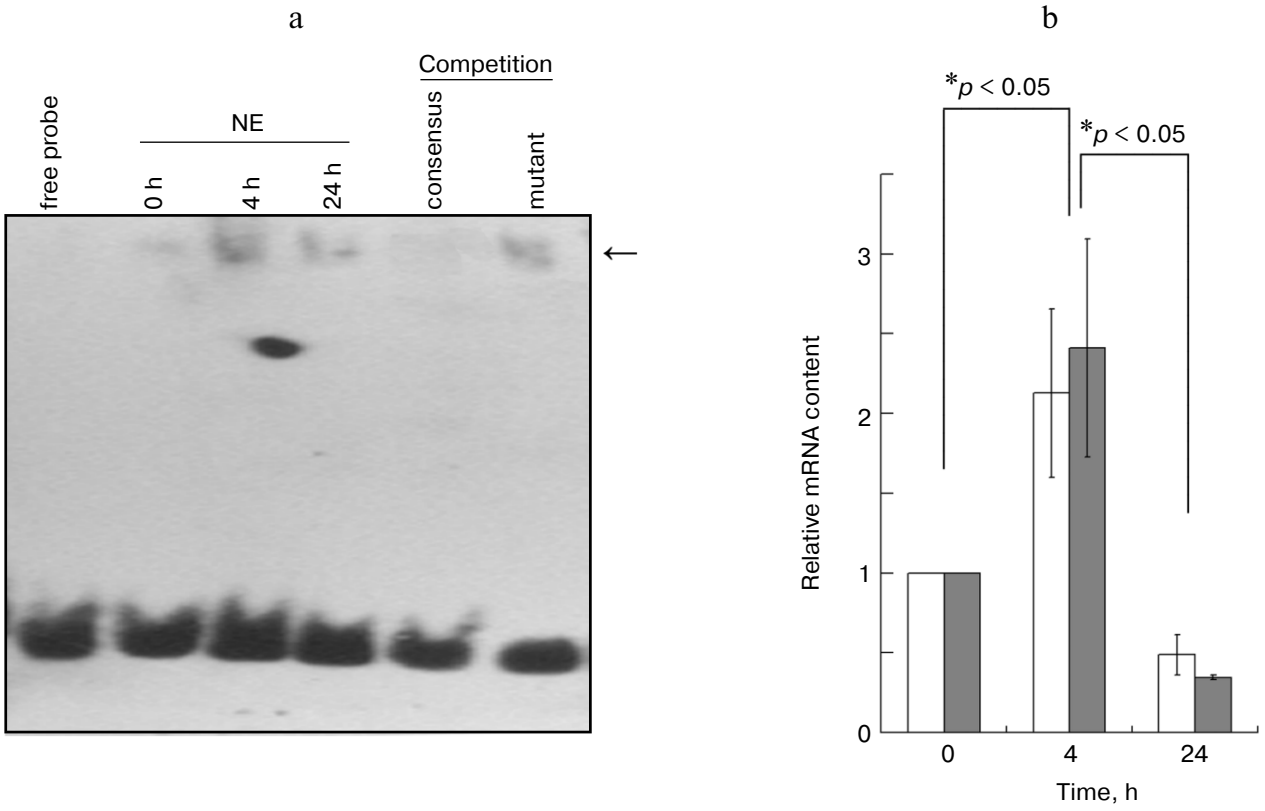

Fig. 3. KA-stimulated DNA-binding activity of NF-κB (a) and antiapoptotic gene expression (b). Primary cultured cortical neurons were unexposed (0 h) or exposed (4 or 24 h) to 100 μM KA. a) Nuclear extracts (NE) were prepared as described in "Materials and Methods". b) Here and in Figs. 4c and 5c, light and dark columns correspond to Bcl-xL and XIAP, respectively.

consensus inhibited formation of the complexes, whereas the mutant could not (Fig. 3a). Intriguingly, KA exposure at early stage (4 h) led to marked upregulation of Bcl-xL and XIAP mRNA, revealed by real-time PCR (Fig. 3b). Both molecules are well known to be NF-κB targets and antiapoptotic. These data indicate that KA-induced activation of the NF-κB factor is associated with the activation of antiapoptotic genes, which is a crucial component of neuronal survival signaling pathway at the early stage of response to KA exposure.

Promotion of kainic acid-induced neuronal death by knockdown of p65. It has been argued against the idea that induction of NF-κB activity has neuroprotective effect [20-22]. Since a fast and transient nuclear translocation of p65 was observed (Fig. 2), we attempted to ascertain the role of early NF-κB activation in KA-induced excitotoxicity. For this purpose, we made a pSuper-p65 plasmid for knockdown of p65. Transient transfection and Western blotting showed that transfection of the plasmid could inhibit the expression of endogenous p65 in cultured cortical neurons (Fig. 4a). Knockdown of p65 did not affect the cell viability in cultured neurons, but caused a marked reduction of cell survival in KA exposed neurons (Fig. 4b), indicating that knockdown of p65 can sensitize neurons to KA-induced excitotoxicity. Consistent with this observation, we could not detect KA-induced upregulation of Bcl-xL or XIAP mRNA in p65-knocked down neurons (Fig. 4c). These results indicate that NF-κB activity can regulate a survival response through activating antiapoptotic signal during KA-induced excitotoxicity.

Inhibition of NF-κB activation by calcium influx blockers and antioxidant. Since NF-κB can be induced by "non-inflammatory" activator such as oxidative stress [9], and Ca^{2+} entry through either NMDA channels or Ca^{2+}-AMPA/KA channels can trigger mitochondrial ROS production [27], we thus investigated whether blocking calcium influx and ROS production could inhibit NF-κB activation. KA (100 μM) alone caused a >2-fold increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) within 260 sec after KA stimulation, compared with the basal levels (before stimulation) (Fig. 5a). In response to KA plus CTZ (30 μM), a desensitization blocker of AMPA receptor, Ca^{2+} influx increased to about 4-fold of basal level. KA-increased $[Ca^{2+}]_i$ was blocked by NBQX (5 μM), a selective and competitive AMPA/KA receptor antagonist, or by the membrane permeable calcium chelator BAPTA-AM (50 μM), but not by D-AP5

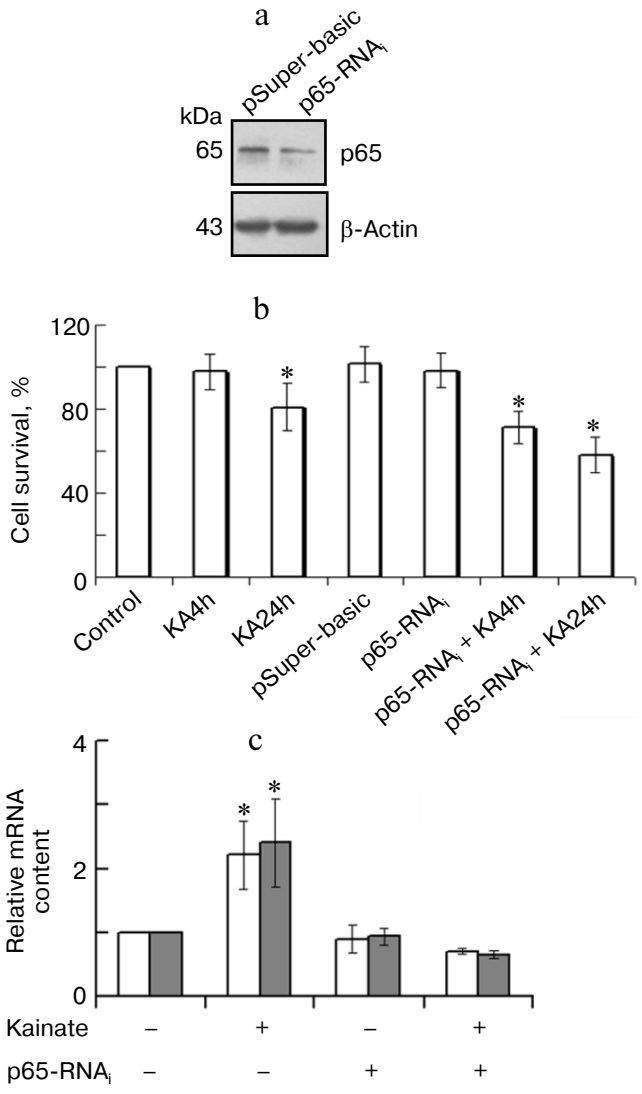

Fig. 4. Decreases in neuronal viability and antiapoptotic gene expression by knockdown of the NF-κB p65 subunit. a) Decrease in the NF-κB p65 subunit protein in neurons by RNA interference (RNAi). Cortical neurons were transfected with pSuper-p65 or pSuper-basic vector plasmids. After transfected cells were cultured for 24 h, Western blotting analysis of p65 was performed using anti-p65 antibody. β-Actin was used as a loading control. b) Decrease in neuronal viability by RNAi of p65. After RNAi of p65, neurons were cultured and exposed to KA for 24 h. Cell viability was analyzed by the MTT method. c) Suppression of endogenous NF-κB targeted genes by RNAi of p65. After RNAi, the neurons were exposed to KA for 24 h. mRNA of Bcl-xL and XIAP was quantified by real-time RT-PCR.

(10 μM), a selective NMDA receptor antagonist. These results indicate that the Ca^{2+} influx induced by KA is mainly mediated by AMPA/KA receptors, which can be abolished by NBQX and BAPTA-AM. To explore whether NF-κB activation was correlated to Ca^{2+} entry and ROS production, EMSA experiments were performed (Fig. 5b). An increase in DNA–nucleoprotein complex was observed when the NF-κB binding consen-

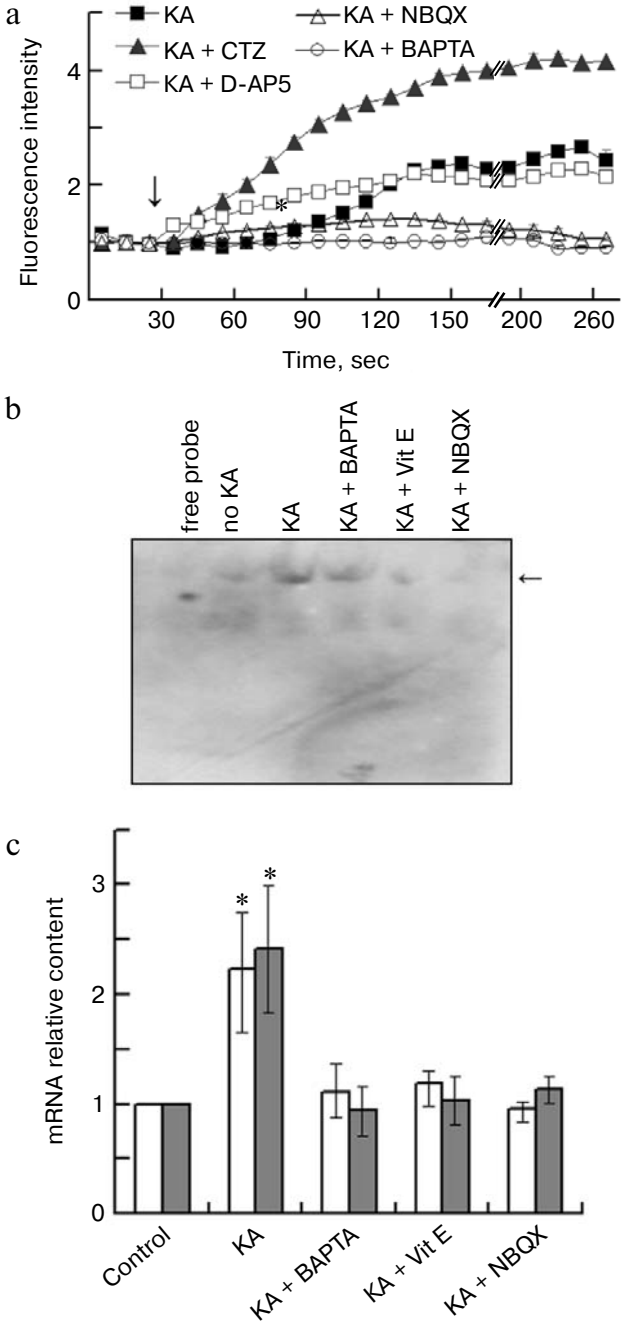

Fig. 5. Implication of ROS and calcium signals in KA-induced NF-κB activity. a) $[Ca^{2+}]_i$ changes stimulated by KA. Cortical neurons were preincubated for 30 min in medium containing D-AP5 (10 μM), NBQX (5 μM), CTZ (30 μM), or BAPTA-AM (50 μM). Fluo-3 fluorescence was recorded for at least 30 sec before and 300 sec after KA stimulation. Image analysis was carried out with a laser confocal scanning microscope-assisted multiple excitation magical imaging system. Arrow indicates the time for KA stimulation; $n = 16$-24. b) Inhibition of DNA-binding activity of NF-κB by antioxidant and Ca^{2+} influx inhibitors. Neurons were exposed to KA plus BAPTA-AM, vitamin E, or NBQX for 4 h. NF-κB DNA-binding activity was analyzed by EMSA using a biotin-labeled probe containing a NF-κB binding consensus sequence. Arrow indicates the DNA–NF-κB complex. c) Inhibition of KA-stimulated Bcl-xL and XIAP mRNA transcription by antioxidant and Ca^{2+} influx inhibitors. The neurons were treated as described in (b). Bcl-xL and XIAP transcripts were quantified by real-time RT-PCR.

sus-containing probe was incubated with nuclear extracts from 4 h KA-exposed neurons. Preloading the cells with BAPTA-AM (50 μM) or NBQX (5 μM) attenuated KA-mediated increases in DNA–nucleoprotein complex formation. Notably, the antioxidant vitamin E could also block KA-stimulated NF-κB binding activity. These data indicate that the activation of NF-κB by KA is associated with calcium influx and ROS production. Consistent with KA-increased NF-κB DNA-binding ability, Bcl-xL and XIAP mRNAs were greatly increased at 4 h after exposure to KA. Moreover, BAPTA-AM and NBQX eliminated the elevation of Bcl-xL and XIAP mRNAs (Fig. 5c). These results suggest that ROS and calcium signals are implicated in KA-induced NF-κB activity.

DISCUSSION

Although the functions of NF-κB in the nervous system under pathological conditions have been widely studied, its neuroprotective role is controversial. We demonstrate that KA can trigger a fast but transient nuclear translocation of the NF-κB p65 subunit at the early stages of KA exposure, which is initiated by calcium signaling. The transient NF-κB activity is associated with upregulated expression of antiapoptotic *Bcl-xL* and *XIAP* genes, thereby protecting against KA-induced neuronal death. Our data suggest that early activation of NF-κB is an important proximal event in response to KA-induced excitotoxicity, which can protect against apoptotic neuronal death.

Constitutive NF-κB activation that is present throughout the developing and adult nervous system plays a physiological role in maintaining survival of central neurons, and further increases in NF-κB activation are neuroprotective [19]. Although the precise stimuli that contribute to constitutive NF-κB activation within neurons are unclear, it is possible that increased NF-κB activation can occur through a paracrine or autocrine activation loop [19, 28]. In addition, neuronal NF-κB can be activated by numerous stimuli, such as glutamate, KA, and nitric oxide [28, 29]. For example, the activity-dependent translocation of p65/RelA from neurites to the nucleus in living neurons can be stimulated by glutamate and KA [28, 30, 31]. These facts raise an intriguing possibility that NF-κB activity can link neuronal activity to cell survival pathways. Activation of NF-κB in neurons promotes neuronal survival by inducing expression of genes encoding antiapoptotic proteins such as Bcl-2, IAPs, and superoxide dismutase [15, 16].

However, others argue against the idea that induction of NF-κB activity is neuroprotective [20-22]. We found that antiapoptotic XIAP and Bcl-xL transcripts were upregulated at the early stage of KA exposure, which was attributed to the nuclear translocation of the NF-κB p65 subunit (Figs. 2 and 3). Furthermore, knockdown of p65 led to inactivation of the *XIAP* and *Bcl-xL* genes, sensitizing cultured neurons to KA-induced cell death (Fig. 4). We therefore suggest that the early activation of NF-κB plays a neuroprotective role in response to KA-induced excitotoxicity. The signal pathways correlated with NF-κB activation in KA-induced excitotoxicity are complicated. The basal levels of activated NF-κB in cortical cultures can be reduced by both MK801 (an antagonist specific to NMDA receptor) and CNQX (an antagonist to AMPA/KA receptors) [32], suggesting that ionotropic glutamate receptors play key roles in the induction of NF-κB [31-33], in which the ability of synaptically released glutamate to activate NF-κB is dependent on simultaneous activation of AMPA/KA receptors, to depolarize the postsynaptic membrane, and NMDA receptors, to initiate the crucial second messenger pathways [32]. Furthermore, KA, but not AMPA or NMDA, has been found to activate NF-κB in rat striatum via calcium influx and p44/p42 MAP kinase activation [34].

It is also suggested that PI3K and ERK signals are primarily involved in KA-mediated NF-κB activation [17]. Production of ROS is increased during KA-induced excitotoxicity and seizures [1, 2], and NF-κB can therefore be activated by ROS via a classic IKK-dependent pathway [9]. Indeed, vitamin E could reduce KA-induced NF-κB binding to DNA (Fig. 5b). Our result supports the notion that KA-activated NF-κB is coupled with ROS production.

The early and transient activation of NF-κB in rat brain has been described in models of ischemia [21, 35, 36], brain tolerance/preconditioning [18], KA-induced neurodegeneration [37], and endotoxic shock [38], in which both proapoptotic [21, 35, 38] and antiapoptotic [18, 36, 37] functions are proposed. Thus, it seems that activation of NF-κB can have a dual action in mechanisms controlling cell survival [36]. During KA-induced epileptic as well as ischemic and polyunsaturated fatty acid-induced preconditioning, a rapid neuroprotective activation of NF-κB occurs in the rat hippocampus [18]. Similarly, following KA injection, activation of NF-κB occurs immediately and transiently in rat CA3 pyramidal neurons (2 h) and disappears one day after KA injection, whereas in microglia and astrocytes NF-κB activation is delayed and continuous (1-7 days) [37]. Consistent with these observations, we found that KA induced an early and transiently protective activation of NF-κB in cultured neurons (Figs. 2 and 3).

The previous data [18, 36, 37] and our observation support the idea that an immediate and transient activation of NF-κB by KA stimulation can confer neuroprotection against hippocampal neuron apoptosis.

The mechanisms by which activated NF-κB plays a neuroprotective role in KA-induced excitotoxicity have previously been described. Generally, NF-κB contributes to neuroprotection after excitotoxic exposure *in vivo* by

using κB decoy DNA [39] or by neuron-restricted ablation of the NF-κB-driven gene [15]. For example, increased NF-κB dimers bind specifically to the *bcl*-x promoter in the hippocampus following hypoxia, thereby promoting expression of antiapoptotic Bcl-x [17]. We did find upregulation of antiapoptotic Bcl-xL as well as XIAP in parallel with the early activation of NF-κB during KA exposure (Figs. 3b, 4c, and 5c). An early (6 h) elevated nuclear Bcl-2 in the newborn striatum has also been observed in low-dose ouabain (a cardiac glycoside)-induced neuroprotection against KA-induced apoptosis [40]. An explanation of Bcl-2 neuroprotection could be that antiapoptotic Bcl-2 family members participate in regulation of ROS production as well as mitochondrial calcium balance and membrane permeability [41]. It is well known that BDNF (brain-derived neurotrophic factor) plays an important role in neuronal survival and synaptic plasticity. KA can induce AMP-activated protein kinase (AMPK) activation, and activated AMPK positively regulates KA-induced BDNF expression via NF-κB activation [42]. In addition, NF-κB activation in hippocampal neurons can induce antioxidant Mn-SOD [18]. Together, there are multiple ways to convert KA-stimulated NF-κB signal into neuroprotection against apoptosis in the model of KA-induced excitotoxicity.

Apoptosis is tightly controlled by the ability of the cell to integrate many pro- and antiapoptotic signals into a binary life/death decision [43] depending on the stimulus properties, lesion characters and extents, and cell types and models. In the model of global ischemia, for instance, the role of transient activation of NF-κB within 24 h is responsible for the induction of protective factors in neurons that survive the ischemic insult, whereas the persistent activation of NF-κB in hippocampal neurons leads to induction of proteins that result in neuronal death [36]. We thus suggest that the most important feature of NF-κB-related life/death is the expression status and profile of NF-κB-targeted genes. It is not surprising that the activation of NF-κB is associated with either apoptosis or survival in different circumstance. Abnormal Ca^{2+} influx through glutamate receptor channels contributes to neuronal death in brain disorders [44], to which ROS is crucial [1, 2, 14]. Production of ROS is attributable to Ca^{2+} increases in the cytosol and mitochondria. The ROS-induced NF-κB activation mechanism relies mainly on IKK activation [9]. We have previously observed that KA induces down-regulation of the GluR2 subunit, leading to formation of Ca^{2+}-permeable AMPA/KA receptors in cultured cortical neurons [23] and to neuron death [24]. Recently, we found that KA-increased Ca^{2+} influx and ROS production induced oxidative DNA damage (8-oxoG production) in rat CA1 and CA3 pyramidal neurons (unpublished data). The activity of NF-κB binding to DNA in KA-exposed neurons is reduced by calcium chelator [28, 42] (also see Fig. 5) as well as antioxidant and AMPA/KA receptor antagonist (Fig. 5b); we therefore conclude that the fast and transient activation of NF-κB in KA-exposed neurons is attributed to ROS production and Ca^{2+} influx.

In conclusion, NF-κB transcriptional activity is prominent at an early stage of KA exposure, which can confer neuroprotection against KA-induced neurotoxicity through activating expression of antiapoptotic genes. Our data suggest that the fast and transient activation of NF-κB initiated by ROS and calcium signal is one of the important proximal events in response to KA-induced excitotoxicity. The current finding will increase our understanding of NF-κB-associated cell life/death in KA-induced neuronal toxicity.

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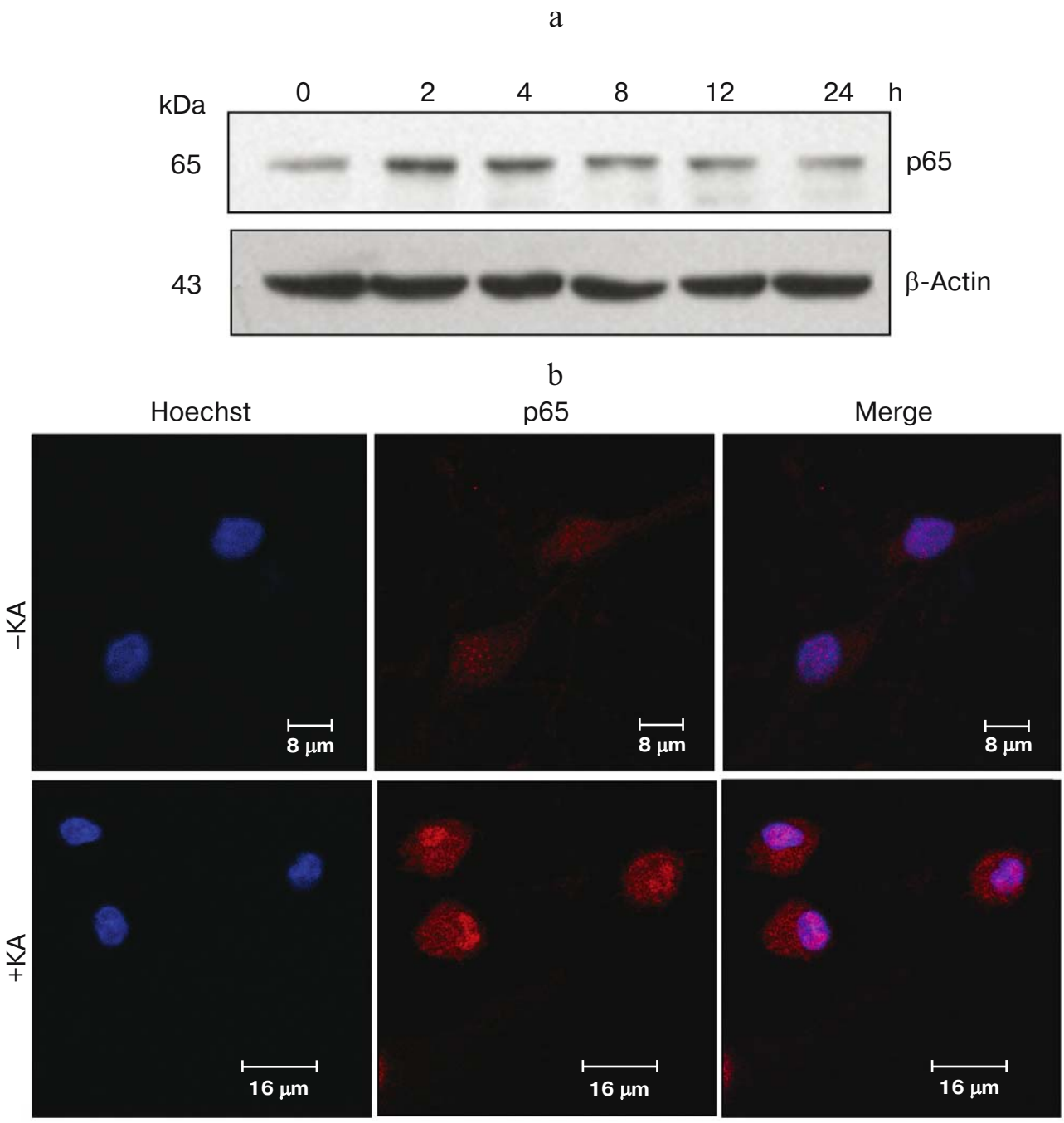

Fig. 2. (Shu-Yan Li et al.) Fast and transient nuclear translocation of the NF-κB p65 subunit after KA exposure. a) Western blotting analysis of the NF-κB p65 subunit in nuclear extracts (NE) from cortical neurons exposed to KA. NE were prepared as described in "Materials and Methods". β-Actin was used as a loading control. b) Immunocytochemical staining showing nuclear translocation of p65 subunit. Primary cultured cortical neurons were unexposed (−KA) or exposed (+KA) to 100 μM KA for 4 h. After being fixed and permeabilized, the cells were incubated with anti-p65 antibody, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (red). Nuclei were stained with Hoechst 33342 (blue). The images were acquired with a laser confocal microscope.