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KR-33028, a Novel Na^+/H^+ Exchanger-1 Inhibitor, Attenuates Glutamate-Induced Apoptotic Cell Death through Maintaining Mitochondrial Function

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Abstract

Preciously, we demonstrated that a novel NHE-1 inhibitor, KR-33028 attenuated cortical neuronal apoptosis induced by glutamate. In the present study, we investigated the signaling mechanism of neuroprotective effect of KR-33028 against glutamate-induced neuronal apoptosis, especially focusing on mitochondrial death pathway. Our data showed that glutamate induces a biphasic rise in mitochondrial Ca^{2+} and that KR-33028 significantly prevents the second phase increase, but not the first phase increase in mitochondrial Ca^{2+}. Furthermore, KR-33028 restored the $\Delta\Psi_m$ dissipation and cytochrome c release into cytoplasm induced by glutamate in a concentration-dependent manner. The inhibition of mitochondrial Ca^{2+} overload by ruthenium red also inhibited glutamate-induced apoptotic cell death, mitochondrial membrane potential, $\Delta\Psi_m$ dissipation and cytochrome c release. These data suggest that inhibition of mitochondrial Ca^{2+} overload is likely to be attributable to anti-apoptotic effect of KR-33028. Taken together, our results suggest that anti-apoptotic effects of NHE-1 inhibitor, KR-33028 may be mediated through maintenance of mitochondrial function.

Key Words: KR-33028, Glutamate excitotoxicity, Na^+/H^+ exchanger-1 (NHE-1), Mitochondria, Apoptosis

INTRODUCTION

Ischemia has been proposed to cause an excess increase in the extracellular concentration of glutamate, an excitotoxic amino acid, in the central nervous system (Nakayama *et al.*, 2002). Glutamate excitotoxicity was triggered by entry of extracellular Ca^{2+} through activation of NMDA receptor of ionotropic glutamate (Choi, 1988). The increase in intracellular Ca^{2+} leads to a cascade of events, which can precipitate necrosis and/or apoptosis of susceptible neurons (Matsumoto *et al.*, 2004). The increase in cytosolic Ca^{2+} is associated with mitochondrial Ca^{2+} loading and slight mitochondrial depolarization followed by profound depolarization concurrent with the loss of ionic homeostasis (Hans *et al.*, 2005). In consequence, excessive Ca^{2+} uptake into mitochondria has been shown to reduce mitochondrial membrane potential ($\Delta\Psi_m$), to release toxin proteins such as cytochrome c and apoptosis inducing factors into cytoplasm through opening of mitochondrial permeability transition pore (mPTP), which may play a central role in the apoptotic pathway (Sullivan *et al.*, 2005).

Cerebral ischemia causes intra- and extra-celluar lactacidosis owing to anaerobic glycolysis and the release of acid equivalents due to hydrolysis of ATP by disturbing the energy metabolism (Glunde *et al.*, 2002). Glutamate also produces intracellular acidosis in cultured neurons (Hartley and Dubinsky, 1993). Na^+/H^+ exchange-1 (NHE-1) is activated by intracellular acidification, leading to Na^+ uptake from the extracellular medium and extrusion of intracellular H^+ (Trudeau *et al.*, 1999). However, excessive activation of NHE-1 during glutamate excitotoxocity leads to remarkable elevation of intracellular Na^+, and then the increase in Na^+ influx reverses the Na^+/Ca^{2+} exchange (NCX) system to protrude Na^+, subsequently causing intracellular Ca^{2+} overload (Matsumoto *et al.*, 2004). Recent investigations on neuronal tissue culture and an animal ischemia model suggested that the blockage of NHE-1 may protect ischemic brain tissue possibly by preventing intracellular Ca^{2+} entry due to NHE-1 inhibition (Glunde *et al.*, 2002). We have recently reported that NHE-1 inhibitors such as sabiporide, zoniporide and KR-33028 [4-subsituted (benzo[b]thiophene-2-carbonyl) guanidines] can inhibit glutamate excitotoxicity

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in vitro and brain ischemia model *in vivo* (Park *et al.*, 2005; Lee *et al.*, 2009). We had also previously shown that NHE-1 inhibitors have good cardioprotective activity against apoptosis, which appeared to be mediated by inhibiting mitochondrial death pathways (Kim *et al.*, 2005). However, mechanism for neuroprotective effect of KR-33028 is not clear. In the present study, we investigated the anti-apoptotic mechanisms of KR-33028 against glutamate-induced neuronal death, especially focusing on mitochondrial death pathway.

MATERIALS AND METHODS

All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals as set by the Animal Care Committee at Ajou University.

Chemicals

KR-33028 were synthesized at Korea Research Institute of Chemical Technology and dissolved in dimethyl sulfoxide (DMSO) before experiments. Glutamate, and cytosine-β-arabinofuranoside (Ara-C) were purchased from Sigma (St. Louis, MO, USA), and L-glutamine was from Bio-Whittaker (Walkersville, MD, USA). MK-801 was purchased from Tocris (Ellisville, MO, USA). All fluorescent dyes were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were purchased from Sigma unless otherwise specified.

Primary culture of cortical neurons

Primary mouse cortical neurons were cultured as described previously (Park *et al.*, 2005). Fetal mouse brains were isolated and cortices were dissected. Cerebral cortices were removed from brains of 14-day-old fetal mice, fetus in the uterus of pregnant (14 day-gestation) mouse, gently titrated 3-4 times with a large-bore Pasteur pipette, dissociated into individual cells using a small-bore Pasteur pipette, and plated on 6- or 24-wells precoated with 100 μg/ml poly-D-lysine (Sigma) and 4 μg/ml natural mouse laminin (Gibco-BRL). The cells from 4.5 hemispheres (approximately 2.5×10^5 cells/plate) were maintained in 10 ml of culture media, consisting of Eagle's minimum essential medium (MEM) (Earle's salts, JBI, Korea) supplemented with 21 mM glucose, 5% fetal bovine serum, 5% horse serum, and 2 mM L-glutamine. Ten μM cytosine arabinofuranoside (Ara-C; Sigma) was added to the cultures at 3-4 days *in vitro* (DIV 3-4) to stop overgrowth of glial cells. The cells were maintained in 5% CO_2 containing atmosphere at 37°C for 7-8 days, and then used for experiments. More than 80% of the cells population at this stage was shown to be neuronal cells, according to NeuN (neuronal nuclei, specific neuronal markers) and GFAP (glial fibrillary acidic protein, glial cell markers) staining (data not shown).

Cell viability assay

The cell viability was evaluated by a colorimetric assay using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma). The 5 mg/ml MTT solution was added to cells grown in 24-well plates. After incubation with MTT solution for 2 h at 37°C, the culture medium was removed. Cells were incubated in solubilization buffer (0.5 N HCl, 5% acetic acid, 50% DMF; pH 7.4) overnight at 37°C. The optical density of each well was measured at 540 nm.

Annexin V-FITC (Annexin V) and propidium iodide (PI) staining

Apoptotic and necrotic cells were detected by double-staining with annexin V (BD Biosciences, San Jose, CA) and PI (Invitrogen). Briefly, media were removed, and cells were washed twice with HCSS (HEPES controlled salt solution; 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, 10 mM NaOH, pH 7.4) and incubated in HCSS containing annexin V (2.5 μg/ml) for 30 min at 37°C. Cells were then washed three times in HCSS and PI (25 μg/ml) was for 10 sec. After rinsing with HCSS, cells were observed under a confocal microscope (Olympus, Japan).

Measurement of mitochondrial Ca^{2+}

To determine mitochondrial Ca^{2+} levels, we used the Ca^{2+} fluorophore dihydro-rhod-2 acetoxymethyl ester (Rhod-2), because it has a net positive charge, which contributes to its selective accumulation in mitochondria. Mitotracker green was used as a mitochondrial indicator marker. This dye binds covalently to the inner mitochondrial membrane and fluoresces independently of mitochondrial Ca^{2+} and membrane potential, and provides an independent means of localizing mitochondria, and thus, of determining the specificity of rhod-2 fluorescence (Trolloinger *et al.*, 1997). Primary cortical neuronal cells grown on microwell glass-bottomed culture dishes were replaced by HCSS containing 2 μM rhod-2. The cells were then incubated for 20 min at 4°C, rinsed with HCSS, incubated in fresh HCSS containing 100 nM mitotracker green for 30 min at 37°C (Sato *et al.*, 2005), and rinsed with HCSS. Images were acquired with a confocal microscope using an excitation wavelength of 488 nm (for mitotracker green) and of 590 nm (for rhod-2), and digitized using FLUOVIEW FV300 software (Olympus).

Measurement of $\Delta\Psi_m$

To measure mitochondrial membrane potentials ($\Delta\Psi_m$), we used the membrane-permeable cationic fluorescent dye rhodamine123 (rhodamine123), because it ha a net positive charge, which accumulated and retained in more negatively charged mitochondria (Emaus *et al.*, 1986). Mitotracker red was used as a mitochondrial indicator marker, it binds covalently to sulfhydryls and is retained by mitochondria after depolarization, and thus provides an independent means of localizing mitochondria. Primary cultures of cortical neurons grown on microwell glass-bottomed culture dishes were loaded with 1 μM rhodamine123 in HCSS and incubated for 30 min at 37°C. After rinsing with HCSS, it was replaced with HCSS containing 100 nM mitotracker red and cells were incubated for 30 min at 37°C. After rinsing with HCSS, images were acquired under a confocal microscope using excitation wavelengths of 488 nm (for rhodamine123) and 590 nm (for mitotracker red). The fluorescence intensity of rhodamine123 was also digitized using FLUOVIEW FV300 software.

Separation of cytoplasmic and mitochondrial fractions and Western immunoblot analysis for cytochrome c release

Cytochrome c release from mitochondria to cytoplasm was assessed after subcellular fractionation as described, with some modifications (Wang *et al.*, 2001). Cells were harvested from six-well plates after incubation with lysis buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose,

1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin and 10 μg/ml aprotinin, pH 7.5) for 1-2 h. The cells were centrifuged at 750 g for 10 min at 4°C to remove nuclear fraction and supernatants were recentrifuged at 10,000 g for 15 min at 4°C. The pellet was used as mitochondrial fraction and supernatant was centrifuged at 100,000 g for 60 min at 4°C. The resulting supernatant was represented as cytoplasmic fraction. To quantify cytochrome c release, the samples were resolved on 15% sodium dodecyl sulfate (SDS) polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA). After incubation with antibodies against cytochrome c (Becton, Dickinson and company, Franklin Lakes, NJ, USA), antibody binding was detected with an enhanced chemiluminescence kit (INTRON Biotechnology, Sungnam, Korea) and quantitated using a LAS1000 (Fuji Photo Film Co., Tokyo, Japan).

Statistical analysis

All data were expressed as mean ± SEM. Multiple comparisons among groups were carried out by one-way ANOVA with Fisher's least significant difference as the post-hoc test, a level of $p<0.05$ was accepted as statistically significant.

RESULTS

Effect of KR-33028 on glutamate-induced neuronal death

To determine whether KR-33028 protects against glutamate-induced cell death, we performed MTT assay. As shown in Fig. 1, cell viability significantly decreased from 100 to 56.3 ± 3.9% after glutamate exposure. Glutamate-induced reduction of cell viability effectively recovered by 100 nM KR-33028 to 93.9 ± 2.0%, 10 μM MK-801 to 98.9 ± 7.7%, and 1 μM DEVD to 78.6 ± 4.6%. Fig. 1A top shows the protective effect of KR-33028 on glutamate-induced cell death by phase contrast morphology. After glutamate exposure, the morphology has changed to that characteristic to neuronal cell death, and this morphological change was recovered by with KR-33028 (100 nM). To examine the neuroprotective effect of KR-33028 on glutamate-induced apoptotic cell death, annexin V/PI staining were performed. Fig. 1B top shows confocal images of cells stained with annexin V (green) and PI (red). Exposure to 100 μM glutamate for 12 h resulted in the appearance of annexin V-positive-PI negative (apoptotic) cells (white arrowhead). The respond to glutamate is characterized by peak in apoptotic cell at 12 h (data not shown). KR-33028 significantly prevented apoptotic cell death in concentration-dependent manners (Fig. 1B). Glutamate-induced apoptotic cell death after 12 h of treated glutamate (44.3 ± 5.2%) was decreased by treatment with 100 nM KR-33028 (20.9 ± 2.5%). In addition, glutamate-induced apoptotic cell death was almost completely blocked by 10 μM MK-801 (17.4 ± 1.1%) and 1 μM DEVD

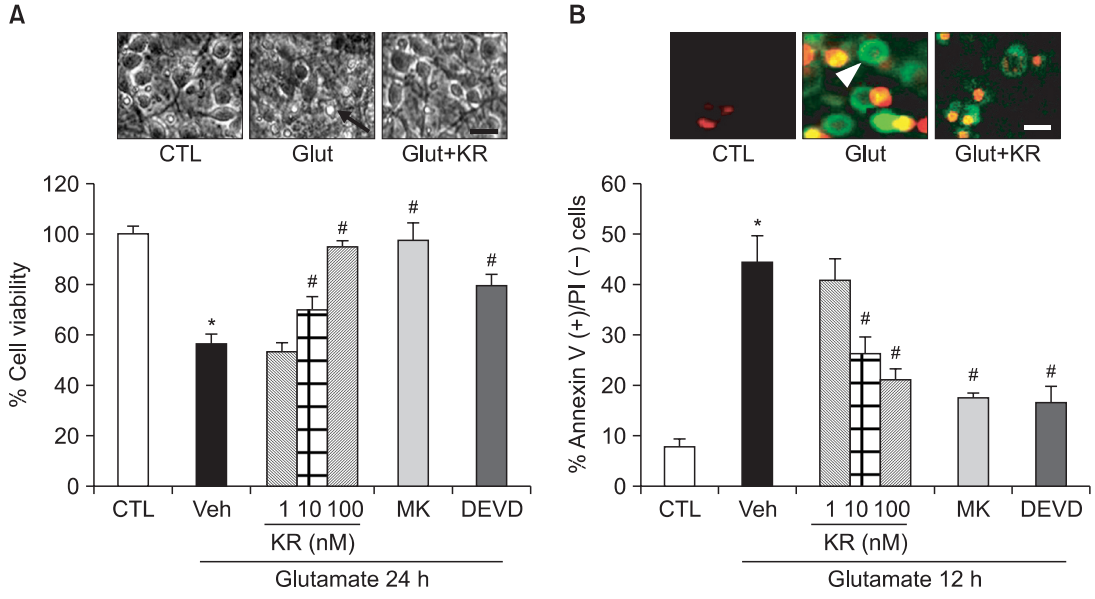

Fig. 1. Effect of KR-33028 on glutamate-induced apoptotic neuronal cell death. (A) Cell viability was measured by MTT assay. Top, morphological change during 100 μM glutamate exposure in the presence and absence of 100 nM KR-33028 was observed at 24 h under the phase-contrast microscope. Black arrow is dead cell. Scale bar, 30 μm. Bottom, glutamate-induced neuronal cells were pretreated with KR-33028 (1, 10 and 100 nM), 10 μM MK-801 or 1 μM Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (DEVD). (B) Cells were stained with annexin V (green) and propidium iodide (PI) (red) as probes for apoptosis, respectively. Top, the majority of annexin V is colocalized with PI as seen by the merged image. Representative images of sequential changes of annexin V and PI fluorescence using confocal microscopy in response to glutamate application at 12 h. Exposure to 100 μM glutamate for 12 h resulted in the appearance of annexin V-positive PI-negative (apoptotic) cells (white arrow head). Scale bar, 30 μm. Bottom, the annexin V-positive PI-negative cells were quantified to see the anti-apoptotic effect of KR-33028 after 12 h of exposed glutamate. Glutamate-induced neuronal cells were pretreated with KR-33028 (1, 10 and 100 nM), 10 μM MK-801 or 1 μM DEVD. Data are mean ± SEMs from 3 independent experiments. *indicates significant difference from control (CTL) and #indicates significant difference from vehicle (Veh) (glutamate alone) at $p<0.05$.

(16.6 ± 3.3%). These data reveal that KR-33028 protects neuronal cells from glutamate-induced apoptotic cell death.

Effect of KR-33028 on glutamate-induced mitochondrial Ca^{2+} release

To evaluate the effect of KR-33028 on glutamate-induced increases in mitochondrial Ca^{2+}, we used the mitochondrial Ca^{2+}-sensitive dye rhod-2 and green mitochondrial tracker. As shown in Fig. 2, glutamate alone induced a biphasic increase in mitochondrial Ca^{2+} followed by a persistent rise. The biphasic increases were almost completely blocked by 3 μM ruthenium red, an inhibitor of mitochondrial Ca^{2+} uniporter (Bae *et al.*, 2003) and 10 μM MK-801, whereas KR-33028 significantly inhibited the second phase only (maximum effect). In addition, the lower concentration of ruthenium red (0.3 and 1 μM) did not block the first phase increase in mitochondrial Ca^{2+} as well as second (data not shown).

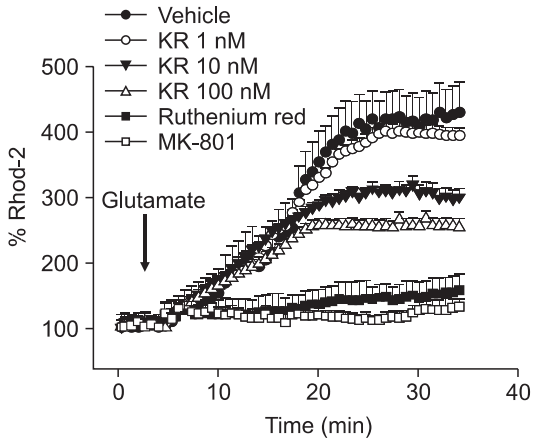

Fig. 2. Effect of KR-33028 on glutamate-induced mitochondrial Ca^{2+} increases. Cells were treated with 2 μM Rhod-2 a probe for mitochondrial Ca^{2+}. Neurons were treated with KR-33028 (1-100 nM), 3 μM ruthenium red or 10 μM MK-801 30 min before exposure to glutamate and glutamate-treated cell maintained for 30 min. Time-dependent changes in the fluorescence of a typical cell exposed to 100 μM glutamate. Results are the means ± SEMs of five separate determinations.

Effect of KR-33028 on glutamate-induced $\Delta\Psi_m$ reduction

We examined whether Ca^{2+} overload into mitochondria induce $\Delta\Psi_m$ reduction and if so, whether $\Delta\Psi_m$ reduction was inhibited with protective effect by KR-33028. As shown in Fig. 3A, we assessed the change of rhodamine123 fluorescence by glutamate in each group using confocal microscope. As shown in Fig. 3B, $\Delta\Psi_m$ significantly fell from 100 to 38.1 ± 1.2% at 30 min after glutamate exposure. Furthermore, glutamate-induced $\Delta\Psi_m$ reduction significantly recovered by 100 nM KR-33028 to 94.2 ± 2.6%, 3 μM ruthenium red to 96.9 ± 3.6%, and 10 μM MK-801 to 97.6 ± 2.8%.

Effect of KR-33028 on glutamate-induced cytochrome c release to cytoplasm

Release of cytochrome c from mitochondria to the cytoplasm is a crucial step in the apoptotic pathway of a cell (Akao *et al.*, 2003). To evaluate the effect of KR-33028 on glutamate-induced cytochrome c release into cytoplasm, we investigated into expression of protein using Western immunoblotting (Fig. 4). Cytochrome c release became evident after glutamate exposure from mitochondria to cytoplasm, and that evidence was abolished by KR-33028 in concentration-dependent manners. COX IV, which is a mitochondrial marker protein, was strongly expressed in the mitochondrial fraction and did not

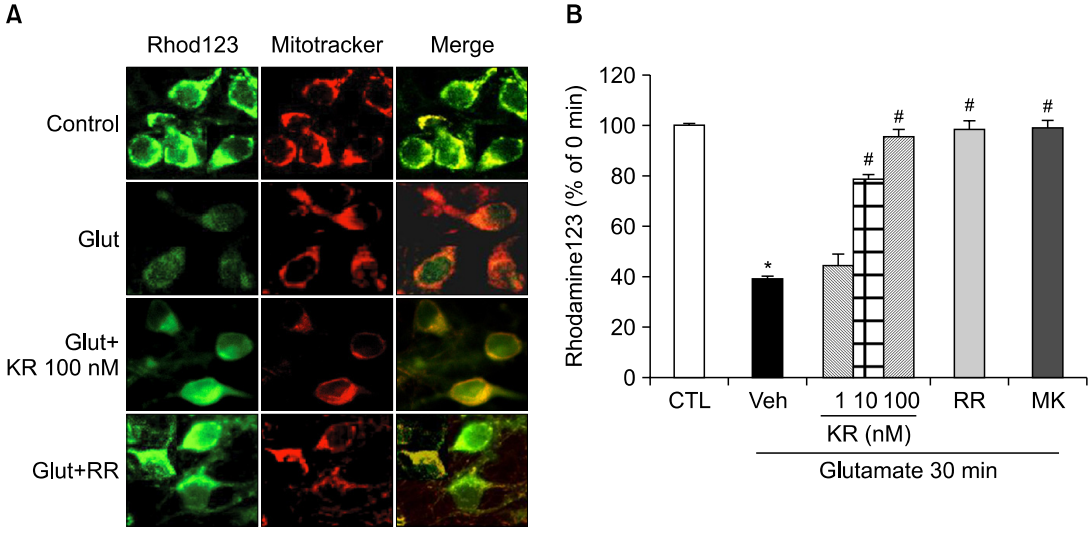

Fig. 3. Effect of KR-33028 on glutamate-induced loss of mitochondrial membrane potential ($\Delta\Psi_m$). Cells were treated with rhodamine123 (Rhod123) as probes for mitochondrial membrane potential ($\Delta\Psi_m$). (A) Confocal micrographs was monitored after labeling rhodamine123 (green) and mitotracker (red) in control, in 100 μM glutamate-treated neuronal cells, and in glutamate-treated cells by treatment with 100 nM KR-33028 or 3 μM ruthenium red (RR) for 30 min. (B) The graph shows mean fluorescence intensity of 20 cells randomly selected in each group. Quantitative data of representative 100 μM glutamate-treated cell undergoing for 30 min in the presence and absence of 1-100 nM KR-33028, 3 μM ruthenium red or 10 μM MK-801. Data represent mean ± SEMs of five individual variations. *indicates significant difference from CTL and #indicates significant difference from Veh at $p<0.05$.

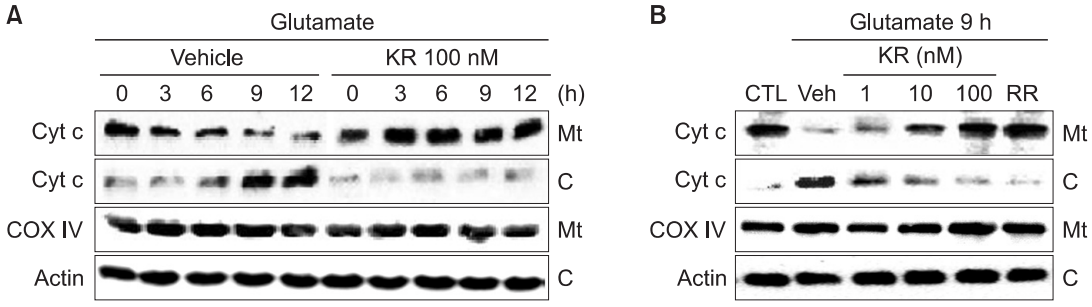

Fig. 4. Effect of KR-33028 on glutamate-induced cytochrome c release from mitochondria to cytoplasm. (A) Primary cultured cortical neurons was exposed to 100 M glutamate in the presence or absence of 100 nM KR-33028 for the indicated times (0-12 h). (B) Primary cultured cortical neurons was exposed to 100 μM glutamate for 9 h in the presence or absence of 1-100 nM KR-33028 (KR) or 3 μM ruthernium red (RR). Mitochondrial (Mt) and cytoplasmic (C) fractions were isolated as described in "Methods". Protein levels of cytochrome c (cyt c) in mitochondria and cytoplasm were determined by Western Blot analysis. Actin and COX IV bands were also used to monitor the same blot to verify consistency of each of cytosolic and mitochondrial protein loading. The content of cytochrome c was measured by LAS pro1000.

decrease after treatment with glutamate, but virtually no band was seen in the cytoplasm fraction. The other way, actin was expressed only in the cytoplasmic fraction and did not express in the mitochondrial fraction because of it is cytoplasmic marker protein.

DISCUSSION

Our previous study has demonstrated that inhibition of NHE-1 with its potent inhibitor KR-33028 attenuates glutamate-induced apoptotic death in neuronal cells (Lee *et al.*, 2009). This study showed that the mechanism for the neuroprotective effect of KR-33028 involves inhibition of mitochondrial death pathways during glutamate excitotoxicity.

Activation of NHE-1 has been reported to contribute to apoptosis in ischemic brain and myocardium (Kim *et al.*, 2005; Wang *et al.*, 2008). Sustained activation of NHE-1 induced by intracellular acidification leads to Na^+ influx from the extracellular medium and extrusion of intracellular H^+ as results of compensative mechanism against deviated intracellular pH levels (Trudeau *et al.*, 1999), which triggers reversal mode operation of NCX and intracellular Ca^{2+} overload (Karmazyn *et al.*, 1999; Pedersen, 2006).This increased cytosolic Ca^{2+} is taken up by mitochondria during glutamate excitotoxicity (Matsumoto *et al.*, 2004; Bano *et al.*, 2005).

Because the mitochondria can take up cytosolic Ca^{2+} rapidly through the potential-driven Ca^{2+} uniporter, they might be neuroprotective by removing Ca^{2+} from the cytoplasm. However, Ca^{2+} uptake into the mitochondria have detrimental effect such as mitochondrial membrane depolarization leading to bioenergetic failure (Stout *et al.*, 1998). In fact, Ca^{2+} is accumulated within mitochondria in neurons exposed to glutamate, and then these events result in cell death *in vitro* and *in vivo* (Zaidan and Sims, 1994; White and Reynolds, 1995). In present study, our data showed that glutamate induces a biphasic rise in mitochondrial Ca^{2+} and that KR-33028 significantly prevented the second phase increase, but not the first phase increase in mitochondrial Ca^{2+} (Fig. 2). These findings suggest that NHE-1 may play a role in the second phase mitochondrial Ca^{2+} excess during glutamate excitotoxicity. From the results that the inhibition of mitochondrial Ca^{2+} overloads by ruthenium red inhibited glutamate-induced apoptotic cell death, it is suggested that an increase in mitochondrial Ca^{2+} concentration may cause neuronal apoptotic death. Furthermore, the anti-apoptotic effects of KR-33028 are also likely to be attributed to the inhibition of second phase mitochondrial Ca^{2+} excess.

Mitochondrial Ca^{2+}-mediated loss of $\Delta\Psi_m$ and release of cytochrome c into cytoplasm are recognized as an important mechanism in apoptotic cell death (Budd and Nicholls, 1996). We previously reported that inhibition of NHE-1 activity by KR-33028 attenuates cardiomyocyte apoptosis during hypoxia through inhibiting mitochondrial death pathways (Jung *et al.*, 2006). Consistent with the previous results, the present study showed that glutamate-induced loss of $\Delta\Psi_m$ and release of cytochrome c into cytoplasm were significantly suppressed by KR-33028. We also observed that the inhibition of mitochondrial Ca^{2+} overload by ruthenium red restored glutamate-induced dissipation of $\Delta\Psi_m$ and cytochrome c release into cytoplasm, which suggests that mitochondrial Ca^{2+} overload may trigger mitochondrial death pathways including dissipation of $\Delta\Psi_m$ and cytochrome c release into cytoplasm.

In summary, these results in this study suggest that the anti-apoptotic effects of KR-33028 may be primarily mediated through inhibiting mitochondrial Ca^{2+} overload and subsequently maintaining mitochondrial function during glutamate excitotoxicity. Furthermore, our results suggest that KR-33028 is likely to be therapeutically beneficial in the treatment of brain ischemia.

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