Research report

\textit{N-Methyl-D-aspartate receptor activation results in regulation of extracellular signal-regulated kinases by protein kinases and phosphatases in glutamate-induced neuronal apoptotic-like death}

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Abstract

Extracellular signal-regulated kinases (ERK1/ERK2) have been shown transiently activated and involved in excitotoxicity. We searched for upstream molecules responsible for the regulation of glutamate-induced ERK1/ERK2 activation and ERK1/ERK2-mediated apoptotic-like death in cultured rat cortical neurons. ERK1/ERK2 activation (monitored by anti-active ERK1/ERK2 antibody) was almost completely prevented by blockage of NMDA receptor (NMDA-R) or elimination of extracellular Ca\textsuperscript{2+}, but not any other glutamate receptor or L-type voltage-gated Ca\textsuperscript{2+} channel. It was prevented largely by inhibition of protein kinase C (PKC), protein-tyrosine kinases (PTK), respectively, but mildly by that of CaM kinase II. Combined inhibition of CaM kinase II (but not PTK) and PKC had an additive effect. Reversion of ERK1/ERK2 activation was largely prevented by inhibition of protein phosphatase (PP) 1 or protein tyrosine phosphatase (PTP). Combined inhibition of PP 1 and PTP had no additive effect. Glutamate-induced apoptotic-like death (determined by DAPI staining) was largely prevented by inhibition of NMDA-R, PKC, CaM kinase II, PTK and MEK1/MEK2 (ERK1/ERK2 kinase), respectively. Combined inhibition of CaM kinase II (but not PKC or PTK) and MEK1/MEK2 had an additive effect. Glutamate-induced apoptotic-like death was promoted by inhibition of PP1 and PTP, respectively. The above results suggested that in glutamate-induced cortical neurotoxicity ERK1/ERK2 activation be mainly mediated by NMDA-R. Subsequently, a pathway dependent on both PKC and PTK was mainly involved, which was also mainly responsible for ERK1/ERK2-mediated apoptotic-like death, and a CaM kinase II-dependent pathway was relatively mildly involved. Reversion of ERK1/ERK2 activation was mainly mediated by a pathway dependent on both PP1 and PTP, which might be involved in the restrain of glutamate-induced neurotoxicity. © 2000 Elsevier Science B.V. All rights reserved.

\textit{Theme: Neurotransmitters, modulators, transporters, and receptors}

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1. Introduction

Glutamate-induced excitotoxicity, with certain characteristics of apoptosis [5,25,48], has been implicated in many neuronal degenerative diseases and considered predominantly mediated by an overload of intracellular Ca\textsuperscript{2+}, mainly as a result of influx via \textit{N}-methyl-\textit{D}-aspartate receptor (NMDA-R) [30,51]. Although the Ca\textsuperscript{2+}-activated intracellular cascades leading to excitotoxicity are largely unknown, potential Ca\textsuperscript{2+} targets include nitric oxide synthase (NOS) [2], protein phosphatases and protein kinases [9,28]. Recently, several molecules including NOS [49,50], protein kinase C (PKC), CaM kinase II [18] and some members of protein tyrosine kinases (PTK), such as PYK2 (proline-rich tyrosine kinase 2) and c-Src [16,29,39], have been shown to transduce Ca\textsuperscript{2+} signaling to ERK1/ERK2 (extracellular signal-regulated kinases) cascade.

ERK1/ERK2, with molecular masses of 44 and 42 kDa, respectively, are classical members of mitogen-activated...
protein kinase (MAPK) superfamily. Both require specific diphosphorylation of both threonine and tyrosine residues at the regulatory sites by MEK1/MEK2 (ERK1/ERK2 kinase) for activation [33,42]. ERK1/ERK2 cascades play important roles in signal transduction from cell surface to nucleus. The well-documented neurotropic growth factor receptor-mediated activation cascade (Ras/Raf/MEK/ERK) has been thought to play important roles in cell growth, proliferation and survival [10,18,20,22,32].

Recently, ERK1/ERK2 have been found activated after relatively mild stimulation of glutamate receptors and involved in some activity-dependent functions [18,20]. Furthermore, ERK1/ERK2 have also been found activated in some excitotoxicity-associated events, such as stroke, seizure and Alzheimer’s disease [3,19,24]. We previously observed that ERK1/ERK2 were transiently activated in glutamate-induced apoptotic-like death in cultured rat cortical neurons, and PD98059, a specific inhibitor for MEK1/MEK2, completely inhibited such activation and partially prevented the glutamate-induced apoptotic-like death [26]. Therefore, ERK1/ERK2 might be excessively activated transiently and involved in the glutamate-induced cortical neurotoxicity.

However, little is known about the upstream cascade of the variation of ERK1/ERK2 in excitotoxicity. In this study, we searched for upstream molecules responsible for the variation of ERK1/ERK2 and the ERK1/ERK2-mediated apoptotic-like death in the glutamate-induced neurotoxicity in cultured rat cortical neurons. Several molecules were investigated, including three subtypes of glutamate receptors, L-type voltage-gated Ca$^{2+}$ channel (L-VGCC), NOS, some protein kinases and protein phosphatases, each of which has been implicated involved either in excitotoxicity or in the regulation of ERK1/ERK2 activation in other cases.

## 2. Materials and methods

### 2.1. Neuronal cultures

Cortical neuronal cultures were prepared from 17-day-old Sprague–Dawley rat embryos as previously described [6]. Briefly, neocortex was meticulously isolated in ice-cold high glucose Dulbecco’s modified Eagle medium (h-DMEM, Gibco-BRL, Grand Island, NY, USA). Cortical cells were dissociated by trypsinization (0.25% (w/v) in protein concentration determination by Lowry method) and seeded onto poly- L -lysine (Sigma, St. Louis, MO, USA)-coated wells or coverslips at a density of 10 cells per cm$^2$, incubated at 37°C for 5 min. Equal amount of proteins (40 μg) were separated by 10% SDS–PAGE by the method of Laemmli [27] and electrotransferred onto nitrocellulose filter (pore size, 0.45 μm, Amersham, Buckingham, UK) by the method of Towbin [46]. The filter was probed with anti-ERK1/ERK2 antibody (Sigma, St. Louis, MO, USA). Cultured cells were rinsed with PBS, scraped off the wells. Each sample was pooled from two wells (approximately 1.2×10$^5$ cells) and homogenized in 160 μl ice-cold buffer (50 mM 3-(N-morpholino) propane-sulfonic acid, MOPS, pH 7.4), 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 0.5 mM EDTA, 1 mM EGTA, 0.5 mM ouabain, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin and 0.5 mM pepstatin A, and centrifuged at 15 000×g for 15 min at 4°C. Ten μl were removed for protein concentration determination by Lowry method [31], the remaining supernatant was incubated in sample buffer (2% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8, and 0.01% bromphenol blue) at 96°C for 5 min. Equal amount of proteins (40 μg) were separated by 10% SDS–PAGE by the method of Laemmli [27] and electrotransferred onto nitrocellulose filter (pore size, 0.45 μm, Amersham, Buckingham, UK) by the method of Towbin [46]. The filter was probed with anti-ERK1/ERK2 antibody (Sigma, St. Louis, MO, USA). Cultures were used after 13 days in vitro when the cells were vulnerable to glutamate insult and verified >95% neurofilaments positive by immunostaining.

### 2.2. Drug treatment

Excitotoxicity was induced by 50 μM glutamate exposure for 15 min, during which the medium was changed into modified EBSS (Mg$^{2+}$-free Earle’s balanced salt solution (EBSS) (Gibco-BRL) supplemented with 5 μM glycine). For restoration, at the end of glutamate exposure, cultures were rinsed twice with EBSS, and the original feeding medium was restored. For drug treatments, PD98059 (Calbiochem, San Diego, CA, USA), MK-801 (dizocilpine maleate, RBI, Natick, MA, USA), L-AP-3 (1-(+)-2-amino-3-phosphono-propionic acid, RBI), DNQX (6,7-dinitroquinoxaline-2,3-(1H,4H)-dione, Sigma), nifedipine (Sigma), EGTA (Sigma), genistein (Sigma), H-89 (Calbiochem), KN-62 (RBI), sphingosine (RBI), L-NNA (N$^\text{3}$-nitro-l-arginine, Sigma) were added for 20 min before till the end of glutamate exposure, respectively, or in combination. In some other cases, sodium orthovanadate (Sigma), okadaic acid (RBI) and cycloporin A (Sigma) were added from 20 min before until 3 h after glutamate exposure, respectively. PD98059, DNQX, nifedipine, genistein, sphingosine, okadaic acid and cycloporin A were made as 500× stocks in dimethyl sulfoxide (DMSO), respectively. KN-62 was made as 300× stocks in methanol. Other drugs were made as 200× stocks in water. Vehicle controls were treated only with 50 μM glutamate and vehicle (0.5% water or 0.2% DMSO or 0.3% methanol) in modified EBSS. Sham controls were treated only with modified EBSS.

### 2.3. Cell extracts preparation and Western immunoblot

Cultured cells were rinsed with PBS, scraped off the wells. Each sample was pooled from two wells (approximately 1.2×10$^5$ cells) and homogenized in 160 μl ice-cold buffer (50 mM 3-(N-morpholino) propane-sulfonic acid, MOPS, pH 7.4), 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 0.5 mM EDTA, 1 mM EGTA, 0.5 mM ouabain, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin and 0.5 mM pepstatin A, and centrifuged at 15 000×g for 15 min at 4°C. Ten μl were removed for protein concentration determination by Lowry method [31], the remaining supernatant was incubated in sample buffer (2% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8, and 0.01% bromphenol blue) at 96°C for 5 min. Equal amount of proteins (40 μg) were separated by 10% SDS–PAGE by the method of Laemmli [27] and electrotransferred onto nitrocellulose filter (pore size, 0.45 μm, Amersham, Buckingham, UK) by the method of Towbin [46]. The filter was probed with anti-ERK1/ERK2 antibody (Sigma, St. Louis, MO, USA). Cultures were used after 13 days in vitro when the cells were vulnerable to glutamate insult and verified >95% neurofilaments positive by immunostaining.
polyclonal, 1:10 000) or anti-active (diphosphorylated) ERK1/ERK2 antibody (Sigma, monoclonal, 1:5000) at 4°C overnight. Detection was carried out by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, 1:20 000) or goat anti-mouse IgG (Sigma, 1: 40 000) and developed with NBT/BCIP color substrate (Sigma). After immunoblot, bands on filter were scanned, quantitative analyzed and printed by an image analyzer affiliated with digital graphic printer (LabWorks Software, UVP, Upland, CA, USA). Protein level and activation (diphosphorylation) level of ERK1/ERK2, based on immunoreactivities of ERK1/ERK2 and active ERK1/ERK2, respectively, were expressed as fold versus sham control of optical density of certain band from Western immunoblot.

2.4. Assessment of apoptotic-like cell death

Live cells grown on each coverslip (2×10^5 cells) were incubated with 10 μg/ml fluorescent DNA binding dye DAPI (4',6-diamidino-2-phenylindole, Sigma) at 37°C for 30 min, washed with PBS and excited with vertical fluorescent at 400 nm. With fluorescence collected at 455 nm, apoptotic-like cells were characterized by the presence of condensed and fragmented nuclei, as opposed to the diffuse staining observed in nonapoptotic cells. Each sample was pooled from three coverslips. The proportion of apoptotic-like cells was calculated as a percentage of total cells counted in 10 microscopic fields (×400).

2.5. Statistics

Values were expressed as mean±S.D. from five independent cultures. One-way ANOVA was used. Comparisons of each group to control were by LSD (least significant difference) test. Others were by q test (Newman–Keuls test). A P value of <0.05 was considered significant.

3. Results

3.1. The role of glutamate receptors, l-VGCC and extracellular Ca^{2+} in ERK1/ERK2 activation in glutamate-induced apoptotic-like death in cultured rat cortical neurons

Protein level of ERK1/ERK2, as indicated by ERK1/ERK2 immunoreactivities, was unaffected (Fig. 1A, top, and B). While activation level of ERK1/ERK2, as indicated by diphosphorylated ERK1/ERK2 (p-ERK1/ERK2), was increased to 4.9-fold versus sham control at 15 min of glutamate exposure (Fig. 1A, bottom, and B). Such activation was almost totally prevented by either blockage of NMDA-R with MK-801 (20 μM) or elimination of extracellular Ca^{2+} with EGTA (5 mM). It was not significantly affected by blockage of mGlu-R with l-AP-3 (1 mM) [41], or KA/AMPA-R with DNQX (20 μM) [23], or l-VGCC with nifedipine (20 μM) [44]. Since the alterations of p-ERK1 and p-ERK2 were much the same, we directed attention to p-ERK2, which displayed a relatively stronger immunoreactivity.

3.2. The role of protein kinases and NOS in ERK1/ERK2 activation in glutamate-induced apoptotic-like death in cultured rat cortical neurons

As shown in Fig. 2, ERK1/ERK2 activation was largely prevented by inhibition of either PTK with genistein (100 μM, also as an inhibitor of topoisomerase II kinase) [1] or PKC with sphingosine (10 μM) [12]. It was weakly but still significantly prevented by inhibition of CaM kinase II with KN-62 (40 μM) [45], but not significantly affected by inhibition of PKA with H-89 (40 μM, also as an inhibitor of PKG) [8], or NOS with l-NNA (100 μM) [11]. The inhibitory effect of KN-62 was significantly weaker than...
Fig. 3. Effects of NMDA-R antagonist, extracellular Ca\textsuperscript{2+} eliminator, inhibitors of NOS and protein kinases on glutamate-induced apoptotic-like death in cultured rat cortical neurons. Thirteen days in vitro cortical neurons were exposed to 50 \mu M glutamate for 15 min. MK-801 (20 \mu M), EGTA (5 mM), l-NNA (20 \mu M), genistein (Gen, 100 \mu M), KN-62 (40 \mu M), sphingosine (SS, 10 \mu M) and PD98059 (PD, 50 \mu M) were added into the medium 20 min before and during the glutamate exposure, respectively. DAPI staining at 18 h after exposure were quantitative represented as percentage of total cells counted in 10 microscopic fields (\times 400). Each point represents mean\pm S.D. of five independent cultures.

\(P<0.05\) versus sham control (Sham, with no drug treatment), \(P<0.05\) versus vehicle control (Vehi, treated only with glutamate and vehicle), \(P<0.05\) versus single drug treatment.

PD98059 (50 \mu M) [26], or NOS with l-NNA (100 \mu M). A significant additive effect was observed in combined inhibition of CaM kinase II (but not PKC or PTK) and ERK1/ERK2. The number of total cells counted in 10 microscopic fields (\times 400) is about 3400\pm 360 cells.

3.4. The role of protein phosphatases in the reversion of ERK1/ERK2 activation in glutamate-induced apoptotic-like death in cultured rat cortical neurons

As shown in Fig. 4, at 3 h after glutamate exposure, ERK1/ERK2 activation reverted to sham control level. Such reversion was largely prevented by inhibition of both protein phosphatase (PP) 1 and PP2A with 300 nM okadaic acid [4], or protein tyrosine phosphatase (PTP) with sodium orthovanadate (200 \mu M, also as an inhibitor of ATPase and alkaline phosphatase) [15], but not significantly affected by PP2A with 5 nM okadaic acid [4], or PP 2B with cyclosporin A (2 \mu M, also as an immunosuppressant) [17]. Combined use of okadaic acid (300 nM) and sodium orthovanadate (200 \mu M) had no significantly additive effect. The concentration of each drug shown in Figs. 1–5, except for okadaic acid (5 nM), was responsible for the maximal effect of the drug (data not shown). Modified EBSS and vehicles had little effect on ERK1/ERK2 activation (data not shown).
Fig. 5. Effects of inhibitors of protein phosphatases on glutamate-induced apoptotic-like death in cultured rat cortical neurons. Thirteen days in vitro cortical neurons were exposed to 50 μM glutamate for 15 min. Okadaic acid (OA, 300 nM) and sodium orthovanadate (Van, 200 μM) were added from 20 min before until 3 h after glutamate exposure, respectively. DAPI staining at 18 h after exposure were quantitative represented as percentage of total cells counted in 10 microscopic fields (×400). Each point represents mean±S.D. of five independent cultures. *P<0.05 versus sham control (Sham, with no drug treatment), †P<0.05 versus vehicle control (Vehi, treated only with glutamate and vehicle), ‡P<0.05 versus single drug treatment.

3.5. The role of PP1 and PTP in glutamate-induced excitotoxicity in cultured rat cortical neurons

As shown in Fig. 5, inhibition of PP1 with 300 nM okadaic acid and PTP with sodium orthovanadate (200 μM) can promote glutamate-induced apoptotic-like death from 82 to 93 and 96%, respectively. Modified EBSS and vehicles had little effect on apoptotic-like death (data not shown).

4. Discussion

We have previously shown that ERK1/ERK2 were transiently activated in glutamate-induced apoptotic-like death in cultured rat cortical neurons, and such activation was almost completely prevented by either blockage of NMDA-R or elimination of extracellular Ca²⁺ [26]. L-VGCC and glutamate receptors, including NMDA-R, α- amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate receptor (AMPA/KA-R) and metabotropic glutamate receptor (mGlu-R), have all been shown involved in ERK1/ERK2 activation in some other cases [18,20]. However, in this study, blockage of none of them, except for NMDA-R, significantly affected ERK1/ERK2 activation. Therefore, in glutamate-induced cortical neurotoxicity ERK1/ERK2 activation might be mainly mediated by NMDA-R-induced influx of extracellular Ca²⁺.

Stimulation of NMDA-R has been shown to mediate ERK1/ERK2 activation, which might involve PKC and CaM kinase II in hippocampal neurons [18] and PTK in striatal neurons [47]. However, the upstream cascade(s) is unknown in excitotoxicity. In this study, in glutamate-induced cortical neurotoxicity ERK1/ERK2 activation was largely prevented by inhibition of PKC, relatively mildly prevented by that of CaM kinase II, and combined inhibition of these two had an additive and complete inhibitory effect. These results strongly indicate that in ERK1/ERK2 activation a PKC-dependent pathway was mainly involved, a CaM kinase II-dependent pathway was relatively mildly involved, and these two pathways were relatively independent on each other. Moreover, such activation was largely prevented by inhibition of PTK and no significant additive effect was observed in combined
inhibition of PTK and PKC. Therefore, PKC and the PTK might act on the same pathway largely contributing to ERK1/ERK2 activation. The exact relationship between PTK and PKC is unclear. However, two pathways may exist. One may be PKC–c-Src pathway. Schlaepfer et al. has shown that PKC-mediated c-Src activity played a role in integrin-induced ERK1/ERK2 activation in NIH 3T3 fibroblasts [40]. And the other may be PKC–PYK2 pathway. Treatment of PC12 cells with agents that increased intracellular Ca$^{2+}$-or activated PKC-stimulated PYK2 activity, and overexpression of PYK2 increased ERK1/ERK2 activity in these cells [29]. Thus, an important connection linking Ca$^{2+}$, PKC, PYK2 and ERK1/ERK2 may be present in some neuronal cells.

CAMP-dependent protein kinase (PKA) has been shown activated after NMDA-R [7], although there are numerous examples of antagonism and synergism between Ca$^{2+}$ and cAMP signaling. PKA has also been shown to interfere with ERK1/ERK2 activation in many cell types, although the interactions between cAMP and ERK1/ERK2 signaling system are complex and variable [20]. However, our results suggest that PKA be not involved in ERK1/ERK2 activation in excitotoxicity.

Another possible mediator of ERK1/ERK2 activation in excitotoxicity is NOS. NOS-produced nitric oxide (NO) has been shown involved not only in glutamate-induced ERK1/ERK2 activation [49,50] but also in excitotoxicity [2,36]. Unexpectedly, we did not observed any effect of l-NNA, a NOS inhibitor, on ERK1/ERK2 activation in glutamate-induced cortical neurotoxicity, although we found that l-NNA provided significant neuroprotection against the glutamate-induced excitotoxicity. Our finding is contrary to that by Yun et al. [50]. The basis for this discrepancy is unclear, but presumably reflects differences in culture conditions. We used a serum-free medium specific to neuron culture, while they used a serum-containing medium. In fact, the time course of ERK1/ERK2 activation in our study is different from theirs. This may also reflects the different mechanism in ERK1/ERK2 activation. They found that ERK1/ERK2 is not fully activated until 10 min after either NO, or NMDA, or glutamate treatment, while we found that ERK1/ERK2 activation peaked during glutamate treatment and was rapidly recovered after glutamate treatment. The time course of ERK1/ERK2 activation in our study is consistent with that reported by Fukunaga and Miyamoto [18].

We have previously observed that ERK1/ERK2 activation was involved in the glutamate-induced cortical neurotoxicity [26]. Some other studies have also shown involvements of PKC, PTK and CaM kinase II in excitotoxicity [13,14,21,37]. Therefore, we determined whether protein kinases, which has been shown involved in ERK1/ERK2 activation in excitotoxicity, are also responsible for ERK1/ERK2-mediated apoptotic-like death. Inhibition of ERK1/ERK2, PKC, PTK and CaM kinase II, respectively, significantly prevented the glutamate-induced apoptotic-like cell death and combined inhibition of CaM kinase II (but not PKC or PTK) and ERK1/ERK2 had a significant additive effect. These results suggest that the ERK1/ERK2-mediated apoptotic-like death was mainly a downstream event of PKC and PTK, but not CaM kinase II, in glutamate-induced cortical neurotoxicity.

We have previously observed that ERK1/ERK2 activation reverted to basal level at 3 h after glutamate exposure in cortical excitotoxicity [26]. Other studies also revealed that PP1, PP2A, PP2B and PTP were involved in the rever- sion of ERK1/ERK2 activation in some other cases [34,35,38,43]. However, little is known about the upstream cascade(s) of the reversion of ERK1/ERK2 activation in excitotoxicity. In the present study, reversion of ERK1/ERK2 activation was largely prevented by 300 nM okadaic acid, supposed to inhibit both PP1 and PP2A [4], but not by 5 nM okadaic acid, supposed to inhibit only PP2A [4]. Thus, PP1, but not PP2A, was mainly involved in reversion of ERK1/ERK2 activation. Moreover, such reversion was also largely prevented by inhibition of PTP and combined inhibition of PP1 and PTP had no significant additive effect. Therefore, PP1 and PTP might act on the same signaling pathway largely contributing to the reversion of ERK1/ERK2 activation in excitotoxicity.

Since we have also showed that either PKC or PTK was mainly involved in ERK1/ERK2-mediated apoptotic-like death in glutamate-induced neurotoxicity, we further determined the role of protein phosphatases in glutamate-induced apoptotic-like death. Our results showed that inhibition of either PP 1 or PTP could not only prevent the reversion of ERK1/ERK2 activation but also promote glutamate-induced apoptosis. This would suggest that either PP 1 or PTP could be involved in the reversion of glutamate-induced neurotoxicity. Therefore, they might have an opposite effect to PKC or PTK on glutamate-induced apoptotic-like death through regulating ERK1/ERK2 activation. However, it is still unclear whether these phosphatases act just the reverse action(s) of those kinases.

In conclusion, we have clearly shown that in glutamate-induced cortical neurotoxicity ERK1/ERK2 activation was mainly mediated by NMDA-R-induced influx of extracellular Ca$^{2+}$. Subsequently, a pathway dependent on both PKC and PTK was mainly involved, which was also mainly responsible for ERK1/ERK2-mediated apoptotic-like death, and a CaM kinase II-dependent pathway was relatively mildly involved. Reversion of ERK1/ERK2 activation was mainly mediated by a pathway dependent on both PP1 and PTP, which might be involved in the reversion of glutamate-induced apoptotic-like death. Further study is ongoing to clarify the exact relationship between PKC and PTK and between PP1 and PTP in regulating ERK1/ERK2-mediated apoptosis, the exact PTK member(s) involved, and target molecules of those protein kinases and phosphatases.
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