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Effect of Kainic Acid on the Phosphorylation of Mitogen Activated Protein Kinases in Rat Hippocampus

Je-Seong Won, Jin-Koo Lee, Seong-Soo Choi, Dong-Keun Song, Sung-Oh Huh, Yung-Hi Kim, and Hong-Won Suh

Department of Pharmacology and Institute of Natural Medicine, College of Medicine, Hallym University, Chuncheon 200−702, Korea

In rat hippocampus, kainic acid (KA; 10 mg/kg; i.p.) increased the phosphorylated forms of ERK1/2 (p-ERK1/2) and Jun kinase1 (p-JNK1), but not p-JNK2 and p38 (p-p38). The preadministration with cycloheximide (CHX; 5 mg/kg; i.p.) inhibited KA-induced increase of p-JNK1, but not p-ERK1/2. Surprisingly, the phosphorylated upstream MAP kinase kinases (p-MKKs) were not correlated with their downstream MAP kinases. The basal p-MKK1/2 levels were completely abolished by KA, which were reversed by CHX. In addition, p-MKK4 and p-MKK3/6 levels were enhanced by CHX alone, but were attenuated by KA. Thus, our results showed that KA increased the p-ERK and p-JNK levels in rat hippocampus, which were not parallel with their classical upstreamal kinases.

Key Words: Kainic acid, Cycloheximide, ERK, JNK, p38, Phosphorylation, MKK, Hippocampus

INTRODUCTION

Kainic acid (KA), a neuro-excitatory/neuro-toxic substance, produces seizure and neural degeneration in the mammalian CNS, and appears to provide a good model for some aspects of human temporal lobe epilepsy. It exerts action by direct excitatory effects as well as reduction of synaptic inhibition and also by modulation of the intrinsic Ca^{2+} and K^{+} conductances. Recently, KA has been implicated in several gene expressions, such as proto-oncogenes (Won et al, 1997), p53 (Sakhi et al, 1994), cytokines (Minami et al, 1991), and neurotrophic factors (Bugra et al, 1994). Furthermore, the change of these local gene expressions have been implicated in hippocampal neuronal cell death or survival processes after seizure activity (Bugra et al, 1994; Sakhi et al, 1994; Pozas et al, 1997).

In hippocampal neuronal cell culture, KA was reported to increase the activities of mitogen activated protein (MAP) kinases, such as extracellular signal-regulated protein kinase (ERK), Jun N-terminal kinase (JNK), and p38 MAP kinase (p38) (DeCoster et al, 1998). Although the physiological roles of KA-induced activation of MAP kinases remained poorly understood the activation of these MAP kinases, in many cells or tissues, has been found to be important in determining whether a cell survives or undergoes apoptosis (Xia et al, 1995), and also in activating of transcriptional factors (Treisman, 1996). Indeed, a recent report showed that disruption of the gene encoding JNK3, a member of the JNK family, in mice rendered the mice to be resistant to the excitotoxicity induced by KA and attenuated KA-induced seizure activity, hippocampal neuron apoptosis, and AP-1 DNA binding activity (Yang et al, 1997).

In mammalian cells, there are several distinct MAP kinase kinases (MKKs) identified; one type, termed MKK1/2, does not phosphorylate or activate p38 or JNK, while two other MKKs, known as MKK3 and MKK4, activate p38 but not ERKs; and MKK4 also activates JNKs (Whitmarsh & Davis, 1996). Furthermore, in spite of crucial roles played by MAP

Corresponding to: Hong-Won Suh, Department of Pharmacology and Institute of Natural Medicine, College of Medicine, Hallym University, 1 Okcheon-dong, Chuncheon 200-702, Korea. (Tel) 82-33-240-1654, (Fax) 82-33-240-1652, (E-mail) hwsuh@hallym.ac.kr.

kinases in KA-evoked excitotoxic effect, neuronal apoptosis, and regulation of transcriptional factors in hippocampus, KA-mediated regulation of MAP kinases activities has been poorly understood. In the present study, therefore, we examined the effect of KA on the levels of active phosphorylated forms of three major MAP kinases (ERK, JNK, and p38) in rat hippocampus and also examined the correlationship between MAP kinases and their corresponding MKKs.

METHODS

Drugs treatment

Male Sprague-Dawley rats weighing 220~280 g were used. Two animals per group were housed in a room maintained at 22±5°C with an alternating 12 hr light-dark cycle. Cycloheximide (CHX) was administered intraperitoneally (i.p.) at a dose of 5 mg/kg (body weight) 30 min prior to the administration of KA. KA was administered i.p. at a dose of 10 mg/kg. All drugs were dissolved in phosphate buffered saline [10 mM potassium phosphate (pH 7.5), 150 mM NaCl]. Animals were sacrificed 1 and 2 hr after KA administration. Their hippocampus were dissected, and kept in −70°C until to be used.

Western immunoblot analysis

To reduce the dephosphorylation of phospho- kinases, the pooled rat hippocampus (n=3/group) was rapidly sonicated in 1×SDS loading buffer and boiled. After centrifugation (15,000×g, 10 min), the concentration of protein was determined with detergent compatible protein assay kit (Bio-Rad). Total cellular proteins (50 μg) were separated by electrophoresis in 12% polyacrylamide gels and electrotransferred onto PVDF filters. After blocking with 3 % skim milk in Tris buffered saline [TBS; 10 mM trizma base (pH 8.0), 150 mM NaCl], they were allowed to interact with antisera against MKK1/2 (New England Bio Lab), MKK3/6 (New England Bio Lab), MKK4 (Santa Cruz, CA), ERK1/2 (New England Bio Lab), p38 (New England Bio Lab), JNK (New England Bio Lab), phospho-MKK1/2 (New England Bio Lab), phospho-MKK3/6 (New England Bio Lab), phospho-MKK4 (New England Bio Lab), phospho-ERK1/2 (New England bio Lab), phospho-p38 (New England Bio Lab), and phospho-JNK (New England Bio Lab) in a blocking buffer for 4 hr at room temperature. Filters were then washed 3 times with TBS containing 0.3% Tween-20 in TBS (TBST) for 5 min and then reacted with anti-rabbit donkey IgG conjugated with horseradish peroxidase for 1 h. After washing 3 times with TBST (3×), the filters were added to ECL-plus solution and exposed to X-ray film.

RESULTS

As shown in Fig. 1, 2, p-ERK1/2 levels in rat hippocampus transiently increased at 1hr, and returned to the basal level at 2 hr, while the basal level of p-p38 was not influenced by the administration of KA (10 mg/kg, i.p.). Total ERK1/2 and p38 protein levels did not change by KA treatment at all time points (Fig. 1, 2). Whether concomitant protein synthesis was involved in the basal or KA-stimulated levels of p-ERK1/2 and p-p38, CHX (5 mg/kg, i.p.), a nonspecific protein synthesis inhibitor, was administered

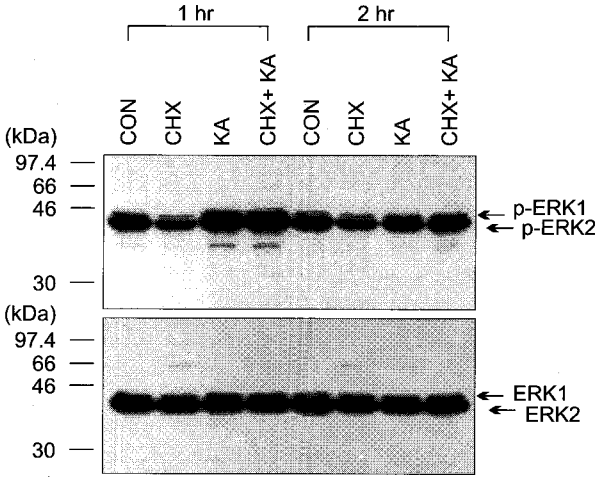

Fig. 1. Effect of kainic acid (KA) and cycloheximide (CHX) on total or phosphorylated extracellular signal-regulated protein kinase1/2 (ERK1/2). After pretreatment with either phosphate buffered saline (PBS) or 5 mg/kg of cycloheximide (CHX) for 30 min, either PBS or 10 mg/kg of KA was administered intraperitoneally. Fifty g of total cellular proteins, which were extracted from pooled rat hippocampus (n=3/group), were used to determine of total ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2). Polyclonal antibodies against ERK1/2 and p-ERK1/2 proteins were used at a 1 : 1,000 dilution.

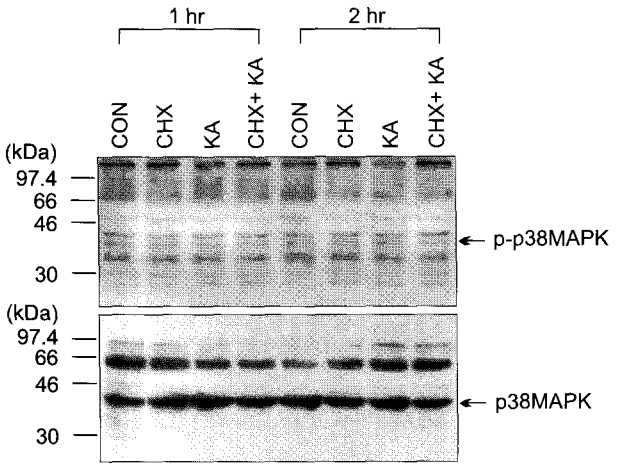

Fig. 2. Effect of kainic acid (KA) and cycloheximide (CHX) on total or phosphorylated p38 mitogen activated protein kinase (p38). After pretreatment with either phosphate buffered saline (PBS) or 5 mg/kg of cycloheximide (CHX) for 30 min, either PBS or 10 mg/kg of KA was administered intraperitoneally. Fifty g of total cellular proteins, which were extracted from pooled rat hippocampus (n=3/group), were used to determine total p38 and p-p38. Polyclonal antibodies against p38 and p-p38 proteins were used at a 1 : 1,000 dilution.

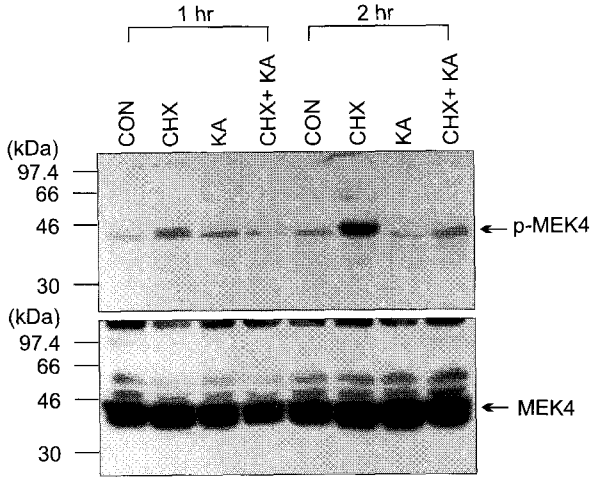

Fig. 4. Effect of kainic acid (KA) and cycloheximide (CHX) on total or phosphorylated mitogen activated protein kinase kinase (MKK)3/6. After pretreatment with either phosphate buffered saline (PBS) or 5 mg/kg of cycloheximide (CHX) for 30 min, either PBS or 10 mg/kg of KA was administered intraperitoneally. Fifty g of total cellular proteins, which were extracted from pooled rat hippocampus (n=3/group), were used to determine total MKK3/6 or p-MKK3/6. Polyclonal antibodies against MKK3/6 and p-MKK3/6 proteins were used at a 1 : 1,000 dilution.

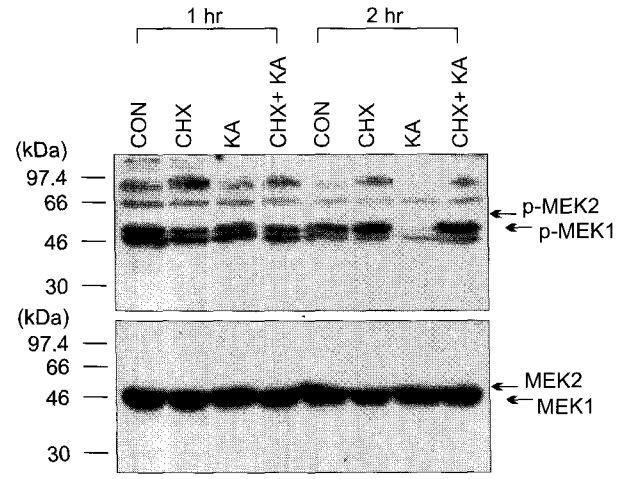

Fig. 3. Effect of kainic acid (KA) and cycloheximide (CHX) on total or phosphorylated mitogen activated protein kinase kinase (MKK)1/2. After pretreatment with either phosphate buffered saline (PBS) or 5 mg/kg of cycloheximide (CHX) for 30 min, either PBS or 10 mg/kg of KA was administered intraperitoneally. Fifty g of total cellular proteins, which were extracted from pooled rat hippocampus (n=3/group), were used to determine total MKK1/2 or p-MKK1/2. Polyclonal antibodies against MKK1/2 and p-MKK1/2 proteins were used at a 1 : 1,000 dilution.

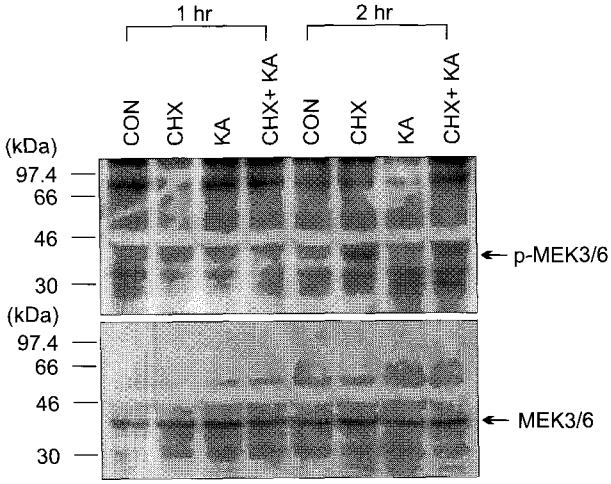

Fig. 5. Effect of kainic acid (KA) and cycloheximide (CHX) on total or phosphorylated mitogen activated protein kinase kinase (MKK)4. After pretreatment with either phosphate buffered saline (PBS) or 5 mg/kg of cycloheximide (CHX) for 30 min, either PBS or 10 mg/kg of KA was administered intraperitoneally. Fifty g of total cellular proteins, which were extracted from pooled rat hippocampus (n=3/group), were used to determine total MKK4 or p-MKK4. Polyclonal antibodies against MKK4 and p-MKK4 proteins were used at a 1 : 1.000 dilution.

30 min prior to KA administration, and CHX was found to cause a reduction of the basal levels of p-ERK1/2.

In contrast to p-ERK1/2 level, CHX, KA, or CHX plus KA did not affect their upstream kinases, p-MKK1/2 levels until 1 hr after the drug treatment, but at 2 hour after KA administration, the basal p-MKK1/2 levels were completely abolished, which were reversed by the pre-administration of CHX (Fig. 3). In addition, p-MKK4 and MKK3/6 levels did not parallel with their downstream kinases, but these p-MKKs were markedly enhanced by CHX administration 2 hr after the drug administration, and these increases were completely blocked by KA (Fig. 4, 5).

DISCUSSION

To understand the effect of convulsant dose of KA on the MAP kinase cascades in the rat hippocampus, the levels of active phosphorylated forme of ERK1/2 and p38 (p-ERK1/2, p-p38) were examined. p-ERK1/2 levels were transiently increased, while the p-p38 was not influenced by the administration of KA. These results are in line with in vitro experiments using primary hippocampal neuronal cell culture, in which KA increased the levels of p-ERK1/2 (De-Coster et al, 1998). However, in hippocampal neuronal cell culture, KA also increased the p-p38 level. Although the mechanism of this phenomenon still remains obscure, it may be due to the intact neuronal circuit or neuronal-glial interaction may inhibit KA-induced activation of p38 in vivo.

To examine the possible involvement of concomitant protein synthesis in the basal or KA-stimulated levels of p-ERK1/2 and p-p38, CHX, a nonspecific protein synthesis inhibitor, was pre-administered. CHX caused a reduction of the basal levels of p-ERK1/2, suggesting that the maintenance of the basal levels of p-ERK1/2 appeared to be tonically regulated by a certain newly synthesized proteins. Recently, we have also reported that KA increased phosphorylation of JNK, especially JNK1, which was attenuated by CHX (Kim et al, 2001).

In many cell types, it has been reported that the activation of ERK is implicated in protection from apoptotic stimuli (Xia et al, 1995; Gardner & Johnson, 1996), or proliferative signals (Pages et al, 1993; Seger & Krebs, 1995; Widmann et al, 1998). In contrast to an ERK pathway, several studies reported that JNK is implicated in apoptotic signaling (Xia et al, 1995; Khwaja & Downward, 1997; Faris et al, 1998; Widmann et al, 1998) via activating or stabilizing the apoptotic factors, such as p53 (Milne et al, 1995; Fuchs et al, 1998). Therefore, the dynamic balance between the activation of ERK and JNK-p38 pathways has been reported to be an important factor in determining whether a cell survives or undergoes apoptosis (Xia et al, 1995). In rat hippocampus, a convulsant dose of KA has been reported to evoke an apoptotic cell death (Pollard et al, 1994), which is prevented by the preadministration of CHX (Schreiber et al, 1993; Sakhi et al, 1994). Therefore, the blockade of p-JNK1 by CHX demonstrated in the present study suggests the protective role of CHX in KA-evoked neuronal cell death. Furthermore, CHX has also been reported to prevent the KA-induced p53 gene expression (mRNA and protein level) (Sakhi et al, 1994), providing a possibility that CHX may also be involved in KA-induced p53 activation via phosphorylation.

There have been several distinct MKKs identified in mammalian cell; one type, termed MKK1/2, does not phosphorylate or activate p38 or JNK while, two other MKKs, known as MKK3 and MKK4, activate p38 but not ERKs, and MKK4 also activates JNKs (Whitmarsh & Davis, 1996). Surprisingly, in this study, the increases of the active phosphorylated forms of these kinases, which were induced by KA or CHX far differed from their downstream kinases. In contrast to p-ERK1/2 level, the basal p-MKK1/2 levels were completely abolished by KA and they were reversed by the inhibition of protein synthesis. In addition, both p-MKK4 and p-MKK3/6 levels were markedly enhanced by CHX administration at 2 hr after the drug administration, and these increases were completely blocked by KA. The exact nature of unparallelism between p-MAP kinases and p-MKKs still remains obscure, but at least two possible contributory mechanisms should be examined to explain this phenomenon. First is the possible involvement of some characterized or uncharacterized upstream kinases in the activation of ERK1/2, JNK, or p38 rather than MKK1/2, MKK4, and MKK3/6, or MAP kinase phosphatases (MKPs). Indeed, in this study, we tested levels of p-MKK1/2, p-MKK4, and p-MKK3/6 only, but our present study suggested that there might be other MKKs such as MKK7, which participated in the regulation of JNK pathway activation (Tournier et al, 1997). In addition, several MKPs including MKP-1,

MKP-2, MKP-3, B23, and PAC1 were also reported to be involved in the inactivation of MAP kinases. Indeed, these phosphatases underwent striking changes in expression within hippocampal subfields CA1-3 and dentate gyrus after KA administration, suggesting that KA-induced expression of MKPs might play a critical role in controlling MAP kinases-dependent processes, including synaptic remodeling and neuronal death (Misra-Press, 1995; Gass et al, 1996; Boschert et al, 1998).

The second possibility is the spatial-temporal difference of activation of MKKs and MAP kinases in hippocampal subfields CA1-4 and dentate gyrus. Indeed, a recent study has shown that according to hippocampal subfields, the distributions of ERK2 and JNK1, and their upstream kinase MEK1 and MEK4 levels are different (Flood et al, 1998). Therefore, to elucidate the parallelism between MKKs and MAP kinases in KA-stimulated rat or mouse hippocampus, we are in process to carry out the immunohistological studies for the short or long time.

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