Natural Product Sciences
15(1) : 32-36 (2009)

Effects of Curcumin, the Active Ingredient of Turmeric (*Curcuma longa*), on Regulation of Glutamate-induced Toxicity and Activation of the Mitogen-activated Protein Kinase Phosphatase-1 (MKP-1) in HT22 Neuronal Cells

Sang-Hyun Lee and Young-Gab Yun*

Department of Oriental Medical Prescription, Wonkwang University, 344-2 Shinyoung-dong, Iksan, Chonbuk 570-749, Korea

Abstract – Glutamate causes neurotoxicity through formation of reactive oxygen species and activation of mitogen-activated protein kinase (MAPK) pathways. MAPK phosphatase-1 (MKP-1) is one of the phosphatases responsible for dephosphorylation/deactivation of three MAPK families: the extracellular signal-regulated kinase-1/2 (ERK-1/2), the c-Jun *N*-terminal kinase-1/2 (JNK-1/2), and the p38 MAPK. In this report, the potential involvement of MKP-1 in neuroprotective effects of curcumin, the active ingredient of turmeric (*Curcuma longa*), was examined using HT22 cells. Glutamate caused cell death and activation of ERK-1/2 but not p38 MAPK or JNK-1/2. Blockage of ERK-1/2 by its inhibitor protected HT22 cells against glutamate-induced toxicity. Curcumin attenuated glutamate-induced cell death and ERK-1/2 activation. Interestingly, curcumin induced MKP-1 activation. In HT22 cells transiently transfected with small interfering RNA against MKP-1, curcumin failed to inhibit glutamate-induced ERK-1/2 activation and to protect HT22 cells from glutamate-induced toxicity. These results suggest that curcumin can attenuate glutamate-induced neurotoxicity by activating MKP-1 which acts as the negative regulator of ERK-1/2. This novel pathway may contribute to and explain at least one of the neuroprotective actions of curcumin.

Keywords – Curcumin, Glutamate, Mitogen-activated protein kinase, Mitogen-activated protein kinase phosphatase-1

Introduction

Curcumin (diferuoylmethane), the active ingredient of turmeric (*Curcuma longa*), has been reported to have a variety of pharmacological activities, including anti-inflammatory, antioxidant, anti-proliferative, and neuroprotective effects (Mattson and Cheng, 2006). Several studies have recently reported that curcumin reduces neurotoxicity induced by a variety of neurotoxins, including beta-amyloid peptide (Smith *et al.*, 2007), kainic acid (Shin *et al.*, 2007) 3-nitropropionic acid (Kumar *et al.*, 2007), 1-methyl-4-phenylpyridinium ion (Chen *et al.*, 2006), glutamate (Wang et al., 2008), and ethanol (Antonio and Druse, 2008). However, the actual neuroprotective mechanisms of curcumin are still not fully understood.

Glutamate is the main excitatory neurotransmitter in the central nervous system. Disturbance of glutamate levels is the primary cause of neuronal death in stroke, mechanical trauma and seizure, and it is considered to play a role in some chronic neurodegenerative disorders, such as Parkinson's or Alzheimer's diseases (Aoyama *et al.*, 2008). Glutamate can induce cell death by two different pathways: excitotoxicity and oxytosis. Excitotoxicity is triggered by the over-activation of glutamate ionotropic receptors and the consequent massive influx of extracellular Ca^{2+}, whereas oxytosis is triggered by the blockade of the cystine/glutamate antiporter, which causes the progressive depletion of the cellular antioxidant glutathione (Aoyama *et al.*, 2008). It is well established that glutamate-induced cell death is related to oxidative stress and potential activation of the mitogen-activated protein kinase (MAPK) pathways (Kang *et al.*, 2007).

Three well-defined MAPK subfamilies include the extracellular signal-regulated kinase-1/2 (ERK-1/2), the c-Jun N-terminal kinase-1/2 (JNK-1/2), and the p38 MAPK. The MAPK pathway is activated through a cascade of sequential phosphorylation events, and inactivated via dephosphorylation of MAPKs mediated by phosphatases. A number of protein phosphatases can inactivate MAPKs, including tyrosine, serine/threonine, and dual-specificity

*Author for correspondence
Tel: +82-63-850-6834; E-mail: yunyg@wku.ac.kr

phosphatases. In mammalian cells, the dual-specificity protein phosphatases, which are often referred to as MAPK phosphatases (MKPs), are the primary phosphatases responsible for dephosphorylation of MAPKs (Boutrose *et al.*, 2008). To date, at least 10 MKPs have been identified in mammalian cells, with MKP-1 being the archetype (Wang and Liu, 2007). Although MKP-1 was initially thought to be a phosphatase specific for the ERK-1/2, recent studies demonstrated that MKP-1 also inactivated JNK-1/2 and p38 MAPK (Wang and Liu, 2007).

Glutamate induces the cell death of HT22 mouse hippocampal cells exclusively through the oxytotic pathway as these cells do not express functional ionotropic receptors (Herrera *et al.*, 2007). Suh *et al.* (2007) have previously described that curcumin prevents glutamate-induced cell death in HT22 cells, but they did not carry out a more profound investigation into the mechanism(s) involved in the protective effect of curcumin. Here, I have reported that curcumin is capable of attenuating glutamate-induced toxicity in HT22 cells through its activation of MKP-1.

Experimental

Materials – Curcumin isolated from the rhizomes of turmeric (*Curcuma longa*), as previously described (Pae *et al.*, 2008). This compound was prepared as 10 mM stock solution in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium was less than 0.1%. Glu, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Dulbecco's-modified Eagle's medium (DMEM, without glutamine) were purchased from Sigma (St. Louis, MO). U0126 and SB203580 were obtained from Tocris (Ellisville, MO). SP600125 was purchased from Calbiochem (san Diego, CA).

Cell cultures and glutamate treatment – The hippocampal cell line (HT22 cells) used in this study is a sub-line cloned from the parent HT4 cells that were immortalized from primary mouse hippocampal neurons using a temperature-sensitive small virus-40 T antigen. HT22 cells were maintained in DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco BRL, Gaithersburg, MD) at 37°C in a 5% CO_2 atmosphere. HT22 cells were pre-incubated for 2 h with curcumin (50 - 200 nM), and then exposed for 24 h to Glu (1 mM) to measure cell viability. Inhibitors of ERK-1/2 (U0126), p38 MAPK (SB203580), and JNK-1/2 (SP600125) were added 30 min before Glu treatment, and then in combination with Glu.

Cell viability measurement – MTT assay was used to determine cellular mitochondrial dehydrogenase activity. Briefly, 24 h after being cultured in 96-well plate, the HT22 cells were treated with curcumin at different concentrations and incubation time. After incubation, cells were treated with the MTT solution (final concentration 0.5 mg/ml) for 4 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO and absorbance at 492 nm was measured with a microplate reader.

Western blot analysis – Cells were lysed in lysis buffer [1% Triton X-100, 1 mM EDTA in 1 x PBS (pH 7.4)] containing 10 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. The lysates were sonicated and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were collected, and the protein concentration of each lysate was determined using the bicinchoninic acid kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Total protein (10 μg) was applied to each lane on 10% SDS-polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride membranes (Millipore, Billerica, MA) were washed in Tris-buffered saline containing 0.1% Tween-20 and then incubated with the following primary antibodies: anti-phospho (p)-ERK-1/2, anti-p-p38 MAPK, anti-p-JNK-1/2 kinase, anti-p-MKP-1, anti-ERK-1/2, anti-p38 MAPK, anti-JNK-1/2, anti-MKP-1 and anti-β-actin (diluted 1 : 1000, Cell Signaling Technology, Beverly, MA). The membrane was then incubated with secondary antibody (1:5000, Pierce, Rockford, IL) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ) was used for detection according to the manufacturer's protocol.

Transfection – MKP-1 small interfering (si) RNA pooled oligonucleotides were purchased from Santa Crutz Biotechnology (Santa Cruz, CA). HT22 cells were plated at 10^5 cells per well in 6-well plates and transfected with MKP-1 siRNA using Oligofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instruction.

Statistical analysis – Data are expressed as mean ± S.E., and the difference was considered statistically significant at $P < 0.05$.

Results

As previously reported (Satoh *et al.*, 2000), glutamate (1 mM) markedly reduced the cell viability of HT22 cells (Fig. 1). Pre-incubation with curcumin at nanomolar concentrations (50 nM - 400 nM) for 2 h attenuated

glutamate-induced cell death (Fig. 1); the maximal protection was observed when the cells were pre-incubated with 200 nM of curcumin, while less than 50 nM of curcumin was not protective (data not shown). Exposure of HT22 cells to glutamate for 3 h or 6 h caused marked activation of ERK-1/2 but not p38 MAPK and JNK-1/2 (Fig. 2A), in agreement with a previous study (Satoh *et al.*, 2000). Pretreatment with curcumin (200 nM) for 2 h significantly suppressed the glutamate-induced activation of ERK-1/2 (Fig. 2B), raising a question as to whether curcumin could protect HT22 cells from glutamate-induced toxicity via its inhibition of ERK-1/2 activation. Indeed, blockade of ERK-1/2 activity by a specific inhibitor (U0125) protected HT22 cells from glutamate-induced cell death (Fig. 2C). However, neither p38 MAPK nor JNK-1/2 inhibitor had any protective effect on glutamate-induced cell death (Fig. 2C).

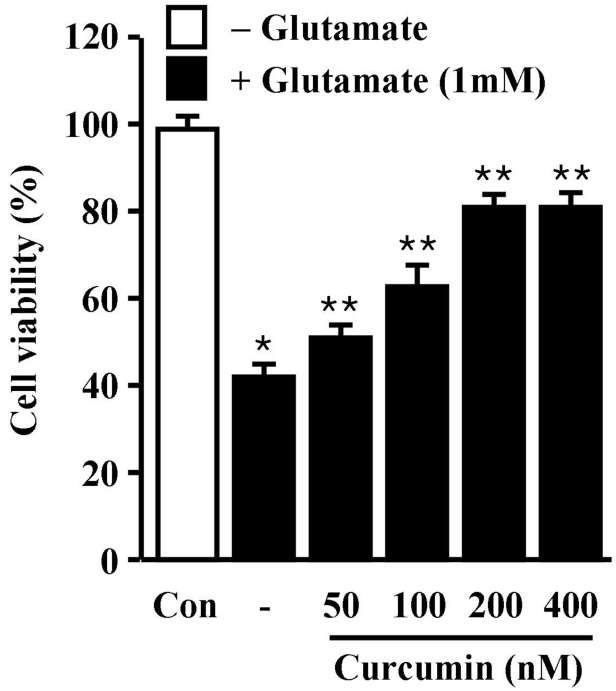

Fig. 1. Effects of curcumin on glutamate-induced cell death in HT22 cells. Cells were pre-incubated for 2 h with indicated concentrations of curcumin, and exposed for 12 h to 1 mM glutamate. Cell viability was determined by MTT assay. $^{*}P < 0.05$ with respect to untreated control group. $^{**}P < 0.05$ with respect to glutamate alone.

MKP-1 has an important role in controlling the magnitude and duration of ERK-1/2 activation (Choi et al., 2006). Thus, this study focused on the possible role of MKP-1 in curcumin-mediated protection. Incubation of HT22 cells with curcumin resulted in time- and dose-dependent phosphorylation of MKP-1 (Fig. 3A and 3B), raising a possibility regarding whether activation of MKP-1 by curcumin would inactivate ERK-1/2 through dephosphorylation of ERK-1/2. For testing this possibility, this study used siRNA to down-regulate cellular synthesis of MKP-1, which led to a failure of curcumin to inhibit ERK-1/2 activation (Fig. 4A). Under these conditions, there was also a failure of curcumin to protect HT22 cells from glutamate-induced toxicity (Fig. 4B).

Discussion

It has been suggested that cell death and survival might be related to activation of ERK-1/2; rapid and transient

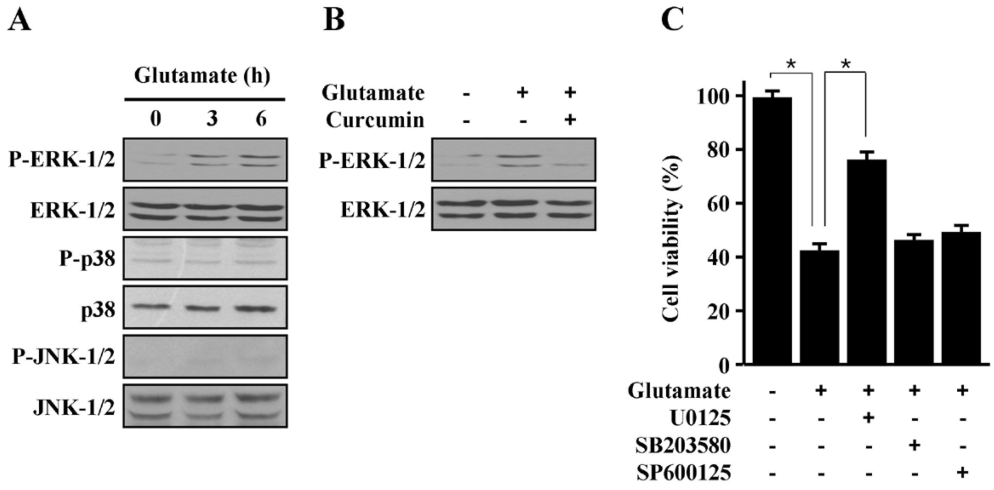

Fig. 2. Effects of curcumin on glutamate-induced activation of ERK-1/2 in HT22 cells. (A) Cells were exposed for 3 h or 6 h to 1 mM glutamate. (B) Cells were pre-incubated for 2 h with 200 nM curcumin, and exposed for 6 h to 1 mM glutamate. (C) Cells were pre-incubated for 1 h with indicated inhibitors, and exposed for 12 h to 1 mM glutamate. Western blot analysis was carried out for detecting ERK-1/2, p38 and JNK-1/2 phosphorylation and each protein, and MTT assay was done for cell viability. $^{*}P < 0.05$.

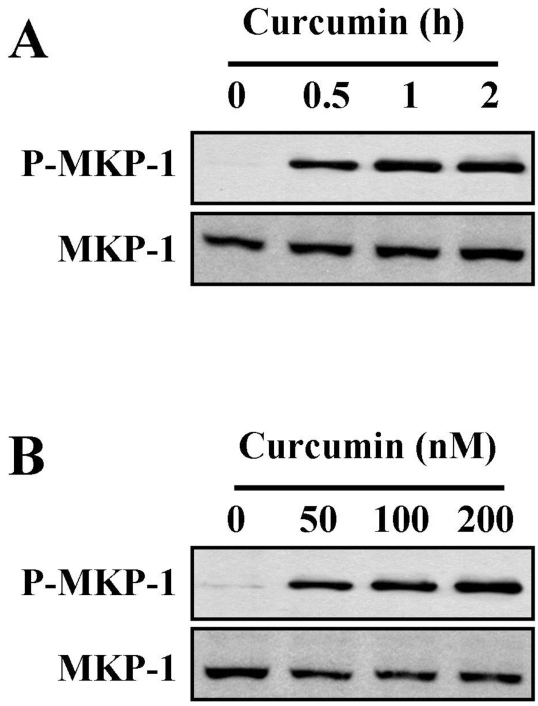

Fig. 3. Effects of curcumin on MKP-1 activation in HT22 cells. (A) Cells were exposed for indicated time periods to 200 nM curcumin. (B) Cells were exposed for 2 h to indicated concentrations of curcumin. Western blot analysis was carried out for detecting MPK-1 phosphorylation and protein.

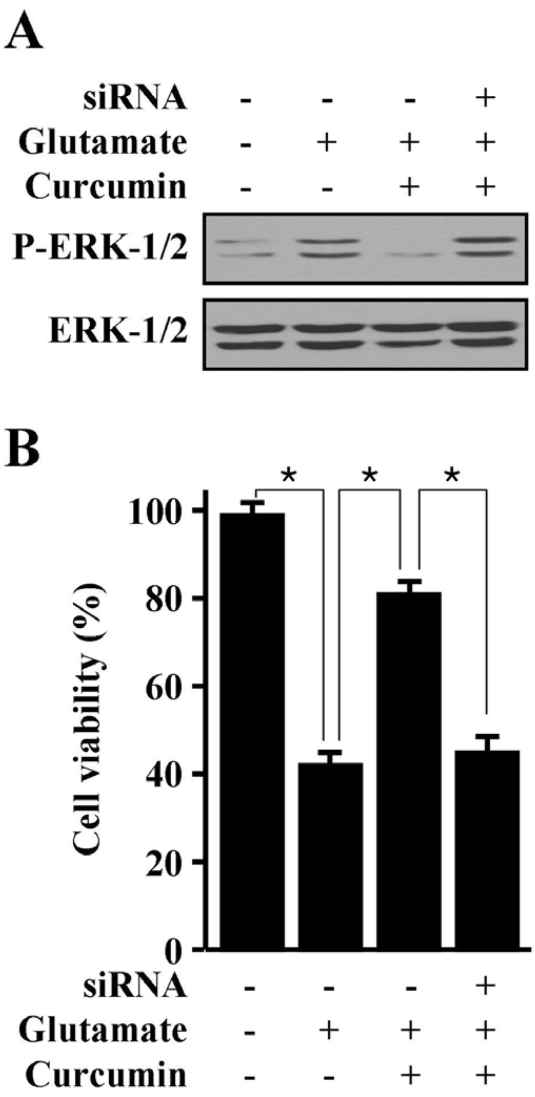

Fig. 4. Roles of MKP-1 in protection by curcumin from glutamate-induced toxicity. Cells were transiently transfected with siRNA against MKP-1, pre-incubated for 2 h with 200 nM curcumin, and exposed for 6 h (A) or 12 h (B) to 1 mM glutamate. Western blot analysis was carried out for detecting ERK-1/2 phosphorylation and protein, and MTT assay was done for cell viability. $^{*}P < 0.05$.

activation of ERK-1/2 has been shown to be associated with enhanced survival response, whereas delayed and sustained activation of ERK-1/2 has a tendency to trigger cell death (Stanciu *et al.*, 2000; Stanciu and DeFranco, 2002). It has been reported that glutamate-induced toxicity in HT22 cells was associated with sustained activation of ERK-1/2 and that inhibition of ERK-1/2 pathway protected the cells against glutamate-induced cell death (Stanciu *et al.*, 2000; Stanciu and DeFranco, 2002). Taking this in mind, it is most likely that curcumin could protect HT22 cells from glutamate-induced toxicity through its inactivation of ERK-1/2 activation.

MKP-1 has been shown to inactivate ERK-1/2, p38 MAPK and JNK-1/2 through dephosphorylation of these kinases (Wang and Liu, 2007), depending on the cell type as well as the cellular context. Studies have demonstrated that the protective effect of MKP-1 was mainly associated with its ability to dephosphorylate ERK-1/2, p38 MAPK or JNK-1/2 (Choi *et al.*, 2006; Besbois-Mouthon *et al.*, 2000). This study has provided evidence supporting that curcumin could induce MKP-1 activation, which was correlated with inactivation of ERK-1/2 that mediates glutamate-induced toxicity. As shown in Fig. 4, knockdown of MKP-1 using siRNA resulted in a failure of curcumin to inhibit ERK-1/2 activation, and abolished the protective effect of curcumin. MKP-1 has also been reported to regulate p38 MAPK and JNK-1/2 activation (Wang and Liu, 2007), and both kinases were previously shown to have crucial roles during oxidative stress in many cell types. However, the phosphorylation of p38 MAPK and JNK-1/2 during glutamate exposure until 6 h was not detected in this study. Moreover, treatment with SB203580 and SP600125, specific inhibitors of p38 MAPK and JNK-1/2, respectively, failed to block the cell death induced by glutamate, excluding p38 MAPK and JNK in

glutamate-induced cell death.

MKP-1 expression can be robustly induced by a variety of extracellular stimuli (Boutros *et al.*, 2008; Wang and Liu, 2007), but the mechanisms underlying its transcriptional activation remain poorly understood. At the post-translational level, MKP-1 activity can be modulated through stabilization of the protein and catalytic activation. The MKP-1 protein can be phosphorylated by its substrate ERK-1/2 at two serine sites in the protein. Thus, it is possible that curcumin may activate transiently ERK-1/2, which, in turn, phosphorylates MKP-1. In addition, MKP-1 protein is degraded by the ubiquitin-proteasome system. It is, therefore, also possible that the ubiquitin-mediated degradation of MKP-1 protein may be inhibited by curcumin. Further studies are required to determine the exact mechanism by which curcumin could activate MKP-1.

In summary, this study has demonstrated, for the first time, that curcumin is capable of attenuating glutamate-induced cell death in HT22 cells by activating MKP-1 which acts as the negative regulator of ERK-1/2 pathway. This novel pathway may contribute to and explain one of the neuroprotective actions of curcumin.

Acknowledgement

This paper was supported by Wonkwang University in 2007.

References

Antonio, A.M. and Druse, M.J., Antioxidants prevent ethanol-associated apoptosis in fetal rhombencephalic neurons, *Brain Res*., **1204**, 16-23 (2008).

Aoyama, K., Watabe, M., and Nakaki, T., Regulation of neuronal glutathione synthesis. *J. Pharmacol. Sci.,* **108**, 227-238 (2008).

Boutros, T., Chevet, E., and Metrakos, P., Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol. Rev.,* **60**, 261-310 (2008).

Chen, J., Tang, X.Q., Zhi, J.L., Cui, Y., Yu, H.M., Tang, E.H., Sun, S.N., Feng, J.Q., and Chen, P.X., Curcumin protects PC12 cells against 1-methyl-4-phenylpyridinium ion-induced apoptosis by bcl-2-mitochondria-ROS-iNOS pathway. *Apoptosis,* **11**, 943-953 (2006).

Choi, B.H., Hur, E.M., Lee, J.H., Jun, D.J., and Kim, K.T., Protein kinase Cdelta-mediated proteasomal degradation of MAP kinase phosphatase-1 contributes to glutamate-induced neuronal cell death. *J. Cell Sci.,* **119**, 1329-1340 (2006).

Desbois-Mouthon, C., Cadoret, A., Blivet-Van Eggelpoel, M.J., Bertrand, F., Caron, M., Atfi, A., Cherqui, G., and Capeau, J., Insulin-mediated cell proliferation and survival involve inhibition of c-Jun *N*-terminal kinases through a phosphatidylinositol 3-kinase- and mitogen-activated protein kinase phosphatase-1-dependent pathway. *Endocrinology,* **141**, 922-931 (2000).

Herrera, F., Martin, V., Garcia-Santos, G., Rodriguez-Blanco, J., Antolin, I., and Rodriguez, C., Melatonin prevents glutamate-induced oxytosis in the HT22 mouse hippocampal cell line through an antioxidant effect specifically targeting mitochondria. *J. Neurochem.,* **100**, 736-746 (2007).

Kang, S., Kim, E.Y., Bahn, Y.J., Chung, J.W., Lee, H.D., Park, S.G., Yoon, T.S., Park, B.C., and Bae, K.H., A proteomic analysis of the effect of MAPK pathway activation on *L*-glutamate-induced neuronal cell death. *Cell Mol. Biol. Lett.,* **12**, 139-147 (2007).

Kumar, P., Padi, S.S., Naidu, P.S., and Kumar, A., Possible neuroprotective mechanisms of curcumin in attenuating 3-nitropropionic acid-induced neurotoxicity. *Methods Find Exp. Clin. Pharmacol.,* **29**, 19-25 (2007).

Mattson, M.P. and Cheng, A., Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. *Trends Neurosci.,* **29**, 632-639 (2006).

Pae, H.O., Jeong, S.O., Kim, H.S., Kim, S.H., Song, Y.S., Kim, S.K., Chai, K.Y., and Chung, H.T., Dimethoxycurcumin, a synthetic curcumin analogue with higher metabolic stability, inhibits NO production, inducible NO synthase expression and NF-kappaB activation in RAW264.7 macrophages activated with LPS. *Mol. Nutr. Food Res.,* **52**, 1082-1091 (2008).

Satoh, T., Nakatsuka, D., Watanabe, Y., Nagata, I., Kikuchi, H., and Namura, S., Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. *Neurosci. Lett.,* **288**, 163-166 (2000).

Shin, H.J., Lee, J.Y., Son, E., Lee, D.H., Kim, H.J., Kang, S.S., Cho, G.J., Choi, W.S., and Roh, G.S., Curcumin attenuates the kainic acid-induced hippocampal cell death in the mice. *Neurosci. Lett*., **416**, 49-54 (2007).

Smith, D.G., Cappai, R., and Barnham, K.J., The redox chemistry of the Alzheimer's disease amyloid beta peptide. *Biochim. Biophys. Acta.,* **1768**, 1976-1990 (2007).

Stanciu, M. and DeFranco, D.B., Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. *J. Biol. Chem.,* **277**, 4010-4017 (2002).

Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angiolieri, M.R., Johnson, J.W., and DeFranco, D.B., Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J. Biol. Chem*., **275**, 12200-12206 (2000).

Suh, H.W., Kang, S., and Kwon, K.S., Curcumin attenuates glutamate-induced HT22 cell death by suppressing MAP kinase signaling. *Mol. Cell Biochem.,* **298**, 187-194 (2007).

Wang, R., Li, Y.B., Li, Y.H., Xu, Y., Wu, H.L., and Li, X.J., Curcumin protects against glutamate excitotoxicity in rat cerebral cortical neurons by increasing brain-derived neurotrophic factor level and activating TrkB, *Brain Res.,* **1210**, 84-91 (2008).

Wang, X., and Liu, Y., Regulation of innate immune response by MAP kinase phosphatase-1. *Cell Signal*., **19**, 1372-1382 (2007).

(Accepted March 5, 2009)