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MIP-2 inhibits β-amyloid peptide (1-42)-mediated hippocampal neuronal apoptosis through activation of mitogen activated protein kinase and phosphatidylinositol 3 kinase signaling pathways

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Running title: CXCR2-mediated signaling in neurons

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Abbreviations used: MAPK, mitogen-activated protein kinase; CREB, cyclic AMP response element binding protein; ERK, extracellular signal regulated kinase; PI3K, phosphatidylinositol 3-Kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PBS, phosphate buffered saline.

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Abstract

β-amyloid peptide accumulation in senile plaques in the brain of Alzheimer's disease patients has been considered as a major cause of neuronal death. The present study demonstrated that the CXCR2 ligands, MIP-2, CXCL1, and CXCL8, protected hippocampal neurons against β-amyloid (1-42) induced death. MIP-2 activated extracellular signal regulated kinase (ERK1/2) and Akt, and both the mitogen activated protein kinase kinase 1 (MEK1) and phosphatidylinositol 3-kinase (PI3K) inhibitors, PD98059 and wortmannin, reduced the neuroprotective effect of MIP-2. MIP-2 induced weak phosphorylation of ribosomal S6 kinase 1 (RSK1), but remarkable phosphorylation and nuclear translocation of RSK2. MIP-2-induced phosphorylation of RSK2 was inhibited by PD98059 but not by wortmannin. MIP-2 treatment of the neuronal cells resulted in phosphorylation of Bad at both the Ser-112 and Ser-136. The phosphorylation at Ser-112 was blocked by PD98059, whereas the phosphorylation at Ser-136 was blocked by wortmannin. The transcription factor, cyclic AMP response element binding protein (CREB), was phosphorylated by MIP-2 stimulation of the neuronal cells. MIP-2-induced CREB phosphorylation was reduced by both PD98059 and wortmannin. These data demonstrate that both MEK1-ERK1/2 and PI3K-Akt signaling pathways are involved in CXCR2-mediated neuroprotection, and multiple downstream signaling events including RSKs, Bad, and CREB, are activated in this process.

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Introduction

Most neurodegenerative diseases are characterized by the progressive loss of neurons in specific brain regions. This is particularly true of Alzheimer's disease (AD), the most frequent cause of dementia (Selkoe DJ, 1997). Several lines of evidence suggest that β-amyloid is involved in the neurodegenerative cascade of AD (Lue *et al*., 1999). The β-amyloid is a potent and direct neurotoxic agent, and it induces a cascade of cellular mechanisms including activation of astrocytes and microglia. It is well known that reactive glial cells produce excessive excitatory amino acids such as glutamate and inflammatory cytokines including interleukin 1, tumor necrosis factor-α, and transforming growth factor β (Popovic *et al*., 1998), that are proposed to play an important role in neuronal death. Moreover, recent studies have demonstrated that reactive glial cells also produce a number of chemokines (Yates *et al*., 2000; Giri *et al*., 2003). So far, immunoreactivity for a number of chemokines (including CXCL1, CXCL8, CXCL10, CCL2, CCL3) and chemokine receptors (including CXCR2, CCR3, CCR5, and CCR1) are found associated with AD pathological changes (Horuk *et al*., 1997; Xia *et al*., 1998; Xia *et al*., 2000; Xia and Hyman, 2002; Halks-Miller *et al*., 2003; Huang *et al*., 2000). Two independent studies demonstrated that CXCR2, the receptor for macrophage inflammatory protein 2 (MIP-2) in mice, CXCL8 and CXCL1, was expressed in neuritic plaques in the brains of AD patients (Xia *et al*., 1997; Huang *et al*., 2000). These studies raise interesting question regarding their role in the CNS.

In contrast to the neurotoxic effects of the excessive glutamate and inflammatory cytokines (Popovic *et al*., 1998), many chemokines in the CNS appear to be

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neuroprotective. Recent studies have revealed that CCL5 prevents β-amyloid peptide (25-35)-induced toxicity in neuronal culture. CXCL8, CCL2, CCL5, and CXCL12 protect neurons against N-methyl-D-aspartate (NMDA)-induced damage (Bruno *et al*., 2000). CCL2, CCL5, CXCL12, and CX3L1 protect hippocampal neurons against gp120 neurotoxicity, which is believed to be the cause of HIV-associated dementia (Meucci *et al*, 1998). All these studies provide evidence for the important role of chemokines in promoting neuronal survival.

Chemokines were primarily identified as molecules that play an important role in migration of leukocytes to the inflammation site. Approximately 40 chemokines have been identified, and they are classified into four subfamilies: CXC, CC, C, and CX3C, based on the number and location of the conserved cysteine residues in the primary structure (Nagasawa *et al*., 1996). Chemokines are able to bind and activate chemokine receptors, which belong to the G protein-coupled receptor superfamily. The binding of chemokines to their cognate receptors triggers a series of G protein-mediated events, including phosphotidylinositide hydrolysis to generate inositol 1,4,5-trisphosphate and diacylglycerol, mobilization of intracellular free Ca^{2+}, as well as activation of mitogen activated protein kinases (MAPKs) (Wu *et al*., 1993). These signaling events play a role in leukocyte migration and in the development of immune system. However, little is known about the mechanisms underlying the neuroprotective effect of chemokines. In the present study, we determined the role of the CXCR2 ligands, MIP-2, CXCL1 and CXCL8 on β-amyloid induced neuronal death, and examined MIP-2, the murine chemokine for CXCR2, induced signaling cascades that are potentially involved in the receptor-mediated protection of mouse hippocampal neurons. The basis for

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investigating the role of CXCR2 in neuroprotection is that CXCR2 appears to be the most strongly expressed chemokine receptor in normal brain and is the only chemokine receptor identified so far in a subpopulation of neuritic plaques in the brain of AD patients (Xia *et al*., 1997; Horuk *et al*., 1997). The CXCR2 ligands, CXCL1, CXCL8 and MIP-2, have been shown to be associated with β-amyloid induced pathogenesis (Xia and Hyman, 2002; Walker *et al*., 2001; Johnstone *et al*., 1999). We demonstrated that the CXCR2 ligands, MIP-2, CXCL1, and CXCL8, significantly inhibited β-amyloid (1-42) induced hippocampal neuronal death. MIP-2 treatment of neuronal cells resulted in activation of MEK-ERK1/2 and PI3K-Akt cascades, leading to the phosphorylation of RSK2, Bad, and CREB. The MEK and PI3K inhibitors significantly attenuated CXCR2-mediated neuroprotection, suggesting involvement of both signaling pathways in this process.

Materials and Methods

Reagents- PD98059 and wortmannin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies directed to p42 MAPK (ERK2, #sc-1647), phospho-p44/p42 MAPK (Tyr-204) (P-ERK1/2, #sc-7383), RSK1 (#sc-231), RSK2 (#sc-9986), phospho-RSK1 (Thr-256/Ser-363, #sc-12898), phospho-RSK2 (Thr-577, #sc-16407), Bad (#sc-8044), phospho-Bad (Ser-112, #sc-7998), phospho-Bad (Ser-136, #sc-7999), CREB (#sc-200), and phospho-CREB (Ser-133, #sc-7978) were all purchased from Santa Cruz Biotechnology, Inc. Hoechst 33258 was purchased from Sigma. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reagents were purchased from Boehringer Mannheim.

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Primary hippocampal neuronal culture. Primary hippocampal cell cultures were established from neonatal mice (born within 24 hours). Dissociated cells were seeded onto poly-L-lysine-coated plastic dishes or 22-mm^2 glass coverslips and incubated in DMEM (GIBCO BRL, Gaithersburg, MD) containing 2 mM L-glutamine, 25 mg/ml gentamicin, 1 mM HEPES, and 0.001% gentamicin sulfate. After 2 days *in vitro* (DIV), non-neuronal cell division was halted by a 3-day exposure to 10 µM β-D-arabinofuranoside. All experiments were performed at 5-7 DIV unless otherwise indicated. Our preliminary experiments using microtubule-associated protein 2 (MAP-2) immunostaining indicated that about 95% of the cells were neurons after β-D-arabinofuranoside treatment for 3 days.

TUNEL assay- The TUNEL assay was performed to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT. Briefly, 1 x 10^5 cells grown in eight-well poly-L-lysine-coated Falcon glass culture slides, were fixed in 4% formaldehyde/PBS (pH 7.4) for 60 min at room temperature, washed in PBS, and then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again, re-suspended in 50 µl of TUNEL reaction mixture or in 50 µl of label solution alone (negative control), and incubated in a humidified dark chamber at 37°C, followed by washing in PBS. The number of TUNEL-positive cells was also counted in five different fields (20×).

Western blot analysis—Hippocampal neurons were incubated with MIP-2 for different time intervals indicated in the figure legends. Cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 20 mM EDTA, 100 µM NaF, 10 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 20 µg/ml aprotinin, and 1% Nonidet P-

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40. Protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Equal amount of proteins were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then exposed to the appropriate antibodies. Phosphorylated ERK1/2, Akt, RSK1 and 2, and CREB were detected with specific antibodies, respectively. Phosphorylated Bad was detected with phospho-specific antibodies against Bad (Ser-112) and Bad (Ser-136), respectively. Total protein levels of ERK2, RSK1, CREB, and Bad were detected with specific antibodies regardless of their phosphorylation state. After incubation with the primary antibodies, the nitrocellulose membranes were incubated with a secondary peroxidase-conjugated antibody. Proteins were visualized with the Amersham ECL system.

Immunofluorescence—Primary hippocampal neuronal cultures were grown on glass coverslips for one day. Neurons were treated with MIP-2 for 1 or 5 min. Following treatment, cells were fixed in methanol, permeabilized in ice-cold 0.2% Triton X-100 in phosphate-buffered saline, and incubated with phospho-RSK2 (Thr-577) antibody overnight at 4 °C followed by incubation with Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst 33258 (1:2000) for 5 min. Coverslips were mounted on slides using the ProLong Antifade Kit (Molecular Probes). Slides were stored at room temperature in the dark until observation.

Statistical Analysis -Data are presented as the means ± S.E. The means of numbers of cells undergoing apoptosis were subjected to analysis of variance for multiple comparisons. Paired analysis between two groups was performed by Student's *t* test.

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Results

MIP2 attenuates β-amyloid induced neuronal apoptosis. Although CXCR2 ligands have been shown to protect neurons against N-methyl-D-aspartate and low concentration of KCl induced death (Bruno *et al*., 2000; Limatola *et al*., 2000), evidence for the neuroprotective effect of CXCR2 ligands on β-amyloid induced neuronal death is lacking. The β-amyloid peptides, such as (1-42) and (25-35) peptides, are known to compromise neuronal survival both *in vitro* and *in vivo.* Studies in Alzheimer's disease have established that high levels of β-amyloid (1-42) result in neuronal cell death via apoptosis and secondary necrosis (Selkoe, 1997). We observed that under our experimental conditions there was a threshold for the effect of β-amyloid (1-42) on cell viability. A 24-hour exposure of hippocampal neurons to β-amyloid (1-42) at 20 μM resulted in significant cell death, but survival was not affected at 1 μM (data not shown). Therefore, a concentration of 50 μM was chosen to induce neuronal death. To determine whether β-amyloid induced neuronal death is reduced by MIP-2 treatment, hippocampal neuronal cultures were incubated with β-amyloid (1-42) peptide (50 μM) in the absence or presence of different concentrations of MIP-2 (0.1-100 nM). Neuronal death was detected by TUNEL assay. As shown in Fig. 1A, β-amyloid (1-42) treatment for 48 hours resulted in remarkable increase in the TUNEL positive cells. Co-administration of MIP-2 dose-dependently reduced the number of the TUNEL positive cells (Fig. 1A). Maximal neuroprotective effect of MIP-2 was observed at the concentration of 10 nM. Fig. 1B shows that β-amyloid (1-42) induced neuronal death in a time-dependent manner, and MIP-2 reduced the neuronal apoptosis in all the time

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points tested. We also tested the effect of other CXCR2 ligands, CXCL1 and CXCL8, on β-amyloid (1-42) induced neuronal death. As shown in Fig. 1C, both CXCL1 and CXCL8 significantly attenuated β-amyloid (1-42)-induced neuronal death as determined by TUNEL assay. To confirm that the ligand-induced neuroprotection acts through CXCR2, antibody neutralization was performed using a mouse monoclonal CXCR2 antibody (E-2, Santa Cruz Biotech.). We observed that addition of the CXCR2 antibody (1:100) to the culture medium, which completely abolished MIP-2 induced ERK phosphorylation (data not shown), significantly blocked CXCL1 or CXCL8 induced neuroprotection (Fig. 1C). In addition, we also determined the effect of CCL5, the specific ligand for CCR5, on β-amyloid (1-42) induced neuronal death by TUNEL assay. As shown in Fig. 1D, CCL5 dose-dependently attenuated β-amyloid (1-42) induced neuronal death, and the maximal neuroprotective effect was observed at 10 nM. These data indicate that CXCR2 and some other chemokine receptors play a role in protecting hippocampal neurons against β-amyloid (1-42) induced cell death.

The neuroprotective effect of MIP-2 requires activation of ERK1/2 and Akt. The stimulation of the mitogen activated protein kinase kinase (MEK)-ERK and PI3K-Akt pathways can result in cell growth and proliferation, differentiation, and cell survival. To understand the molecular mechanisms underlying the neuroprotective effect on β-amyloid (1-42) induced neuronal death, we evaluated the potential activation of the MAPK and PI3K/Akt in neuronal cells in response to MIP-2 treatment. Neurons were treated with MIP-2 for different time intervals. Whole cell lysates were separated by SDS-PAGE and examined by Western blot analysis. Antibodies that specifically react

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with the phosphorylated ERK1/2 and Akt were used to detect the active kinases. For normalization, total ERK2 protein levels were determined with an antibody reacting with both the phosphorylated and non-phosphorylated proteins. MIP-2-induced phosphorylation of ERK1 and ERK2 was noted within 1 min, peaked 2-5 min, and lasted for about 30 min (Fig. 2A and B). However, MIP-2 treatment failed to induce phosphorylation of p38 and JNK MAPKs (data not shown). Stimulation of the neuronal cells also resulted in Akt phosphorylation, which peaked at 5-10 min, and lasted for about 30 min (Fig. 2A and B). To determine whether the MEK1-ERK1/2 pathway and PI3K-Akt pathways are involved in MIP-2-induced neuroprotection, hippocampal neuronal cultures were pre-treated with the MEK1 inhibitor, PD98059 (100 nM) (Salvarezza *et al*., 2003), or the PI3K inhibitor, wortmannin (100 nM) (Salvarezza *et al*., 2003), for 60 min. Cells were exposed to β-amyloid (1-42) peptide (50 μM) in the absence or presence of MIP-2 (10 nM) for 48 hours. Neuronal death was determined by TUNEL assay. As shown in Fig. 2C, pre-treatment of PD98059 or wortmannin significantly attenuated the neuroprotection effect of MIP-2. Pre-treatment of PD98059 or wortmannin also significantly reduced the neuroprotection effect of CXCL1 and CXCL8 (Fig. 2D and E). These data suggest that the neuroprotective effect of MIP-2 and other CXCR2 ligands requires the activation of MEK1-ERK1/2 and PI3K-Akt cascades.

MIP-2 induced phosphorylation of RSKs. The p90 RSKs (RSK1-3) lie at the terminus of the ERK pathway (Salvarezza *et al*., 2003). Activated RSKs play an important role in cell functions including cell survival (Bonni *et al*., 1999). We proposed that stimulation of CXCR2 in neuronal cells may activate RSKs. To test this hypothesis, hippocampal

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neuronal cells were treated with MIP-2 (10 nM) for different lengths of time, and the phosphorylation of RSK1 and RSK2 was detected by Western blotting using antibodies that specifically react with the phospho-RSK1 (Thr-259/Ser-363), and phospho-RSK2 (Thr-577), respectively. For normalization, total RSK1 protein levels were determined with an antibody reacting with both the phosphorylated and nonphosphorylated proteins. As shown in Fig. 3A and B, MIP-2 treatment of the neuronal cells induced modest phosphorylation of RSK1, but strong phosphorylation of RSK2, which peaked at 5 min, and decreased after 15 min. To determine whether the MEK1-ERK1/2 cascade and the PI3K-Akt cascade are involved in MIP-2-induced RSK2 phosphorylation, hippocampal neuronal cultures were pre-treated with either PD98059 (100 nM) or wortmannin (100 nM) for 60 min. Cells were then incubated with MIP-2 (10 nM) for 5 min. The phosphorylation of RSK2 was determined by Western blot analysis described above. As demonstrated in Fig. 3C and D, treatment with PD98059 but not wortmannin blocked the MIP-2-induced RSK2 phosphorylation, suggesting involvement of MEK1-ERK1/2 in the ligand-induced activation of RSK2. We examined the ability of MIP-2 to induce nuclear translocation of RSK2, which is important to modulate the transcription factors. Hippocampal neurons stimulated with 10 nM MIP-2 for 1 or 5 min were double stained with an anti- phospho-RSK2 (Thr-577) antibody to detect the activated RSK2 and Hoechst 33342 to detect nuclei. As shown in Fig. 4, untreated neurons display a diffuse distribution of phosphorylated RSK2, nuclear translocation was noted 1 min after MIP-2 treatment, and after 5 min, high levels of phosphorylated RSK2 were concentrated within the nucleus.

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MIP-2 induced phosphorylation of Bad. Bad has been identified as an intersection point of pro- and anti- apoptotic regulatory cascades. Bad function is modulated by phosphorylation at two sites, Ser-112 and Ser-136 (Del Peso *et al*., 1997). We examined the effect of MIP-2 on the phosphorylation of Bad at Ser-112 and Ser-136. Hippocampal neurons were treated with MIP-2 (10 nM) for different time intervals, and Bad phosphorylation was detected by Western blot analysis using antibodies that specifically react with the phosphorylated Bad (Ser-112) and Bad (Ser-136), respectively. For normalization, total Bad protein levels were determined with an antibody reacting with both the phosphorylated and non-phosphorylated Bad. As shown in Fig. 5A and B, MIP-2 time-dependently induced the phosphorylation of Bad at both the Ser-112 and the Ser-136 residues. The phosphorylation of Bad at both Ser-112 and Ser-136 was detected at 2 min after ligand stimulation. The phosphorylation of Bad at Ser-112 peaked at 5-10 min, whereas the phosphorylation of Bad at Ser-136 peaked at 5 min. Moreover, we confirmed that the total amount of Bad in each lysate was the same by Western blotting with anti-Bad antibody (Fig. 5A and B). The different time course for the phosphorylation of Bad at Ser-112 and Ser136 suggests that Bad is phosphorylated by distinct kinases. This is confirmed by the data showing that MIP-2-induced phosphorylation of Bad at Ser-136 was completely inhibited by wortmannin but was not inhibited by PD98059. On the other hand, MIP-2-induced phosphorylation of Bad at Ser-112 was not inhibited by wortmannin but was completely inhibited by PD98059 (Fig. 5C and D). These data suggest that MIP-2 induced the phosphorylation of Bad at Ser-112 via the MEK1-ERK1/2 cascade and that at Ser-136 via the PI3K-Akt cascade, consistent with the previous reports (Del Peso *et al*., 1997).

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MIP-2 induced phosphorylation of CREB. Since the transcript factor CREB is a downstream factor of the MEK-ERK pathway, we examined whether stimulation of the neuronal cells by MIP-2 results in CREB activation. Time course analysis of CREB phosphorylation induced by MIP-2 revealed that the phosphorylated CREB was detectable after 2 min of stimulation with the peptide. The phosphorylated CREB signal reached peck at 5 min and decreased after stimulation for 10 min (Fig. 6A and B). Analysis of the same protein extracts using another antibody that recognizes CREB independently of its phosphorylated state demonstrated no difference in the total amount of CREB in cells stimulated with MIP-2. We then investigated the possible involvement of ERK- and PI3K-dependent pathways in MIP-2-induced CREB phosphorylation by using specific protein kinase inhibitors. Pre-incubation of hippocampal neuronal cultures for 60 min with the specific MEK1 inhibitor, PD98059, and the PI3K inhibitor, wortmannin, significantly reduced the stimulatory effect of MIP-2 on CREB phosphorylation (Fig. 6C), suggesting involvement of both the MEK1-ERK1/2 and PI3K/Akt pathways in this process.

Discussion

The present study demonstrated that the CXCR2 specific ligands, MIP-2, CXCL1 and CXCL8, protected hippocampal neurons from β-amyloid induced death. Both the MEK1-ERK1/2 and PI3K-Akt pathways are apparently involved in MIP-2-induced neuroprotection. We provide evidence for the first time that the pro-apoptotic protein, Bad, was phosphorylated at both the Ser-112 and Ser-136 sites after MIP-2 treatment, and phosphorylation of Bad (Ser112) involves MEK1-ERK1/2, whereas phosphorylation of Bad (Ser136) involves PI3K-Akt. MIP-2 induced RSK phosphorylation in a MEK1-

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ERK1/2 dependent but PI3K-Akt independent manner. MIP-2 induced phosphorylation and activation of the transcript factor, CREB, and both the MEK1-ERK1/2 and PI3K-Akt pathways are involved in the CXCR2-mediated CREB phosphorylation. Finally, we provided evidence that inhibition of either MEK1 or PI3K resulted in significant attenuation of MIP-2 induced protection against β-amyloid neurotoxicity, suggesting important role of both the MEK1-ERK1/2 and PI3K-Akt pathways in CXCR2-mediated neuroprotection.

We provided evidence that β-amyloid (1-42) treatment of the hippocampal neuronal cultures resulted in significant increase in TUNEL positive cells, suggesting that β-amyloid induces neuronal apoptosis. However, we can not exclude the possibility that β-amyloid also induces neuronal necrosis during the treatment because it has been reported that high levels of β-amyloid induce apoptosis and secondary necrosis in neurons (Selkoe *et al*., 1997), and concurrent apoptotic and necrotic alterations can be observed in the TUNEL positive cells (Wei *et al*., 2004). In addition to the direct induction of neuronal death by the β-amyloid peptide, glutamate and other neurotoxins released from the non-neuronal cells (about 5% neuronal cells in the culture) may also contribute to the compromise of the neuronal viability. Although many chemokines have been reported to protect neurons against different injuries, the protective effects of chemokines on β-amyloid peptide induced neuronal death are not well documented. Our study demonstrated that MIP-2, CXCL1 and CXCL8 attenuated β-amyloid (1-42) induced neuronal death. We postulate that these chemokines protect neurons through CXCR2, because their cognate receptor, CXCR2, is expressed functionally on

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hippocampal neurons (Meucci *et al*., 1998), and neutralizing CXCR2 by a specific antibody significantly blocked the ligand induced neuroprotection. However, it is impossible to rule out the possibility that these chemokines promote neuronal survival by inducing the synthesis or release of another neurotrophic factors. These data are consistent with the previous finding that CCL5, a ligand for CCR5, protects β-amyloid peptide (25-35)-induced neurotoxicity (Bruno *et al*., 2000), but are inconsistent with the result showing that CXCL8 did not exhibit neuroprotective effect on β-amyloid peptide (25-35)-induced death of cortical neurons (Bruno *et al*., 2000). This is likely due to different cell types and different β-amyloid peptides used to induce neuronal death. It has been suggested that different β-amyloid fragments may induce distinct mechanisms of toxicity *in vitro* (Woods *et al*., 1995). Nevertheless, these studies reflect complexity of the functional role of chemokines in CNS, and indicate that the role of chemokines in neuronal viability varies depending on the types of neuronal cells and sorts of neuronal injuries.

The present study demonstrated that MIP-2 activated ERK and PI3K/Akt pathways, consistent with the previous report (Xia *et al*., 2002). Although the CXCR2 ligand, CXCL1, was reported to induce rapid phosphorylation and activation of P38 MAPK in melanoma cell lines (Wang and Richmond, 2001), we did not observe the activation of p38 MAPK or JNK in MIP-2-treated neurons, suggesting that CXCR2 differentially mediates MAPK activation in different cell types. On the other hand, we observed that a sublethal concentration of β-amyloid (1-42) induced JNK but not ERK1/2 and PI3K/Akt activation (our unpublished data). These findings are consistent

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with the hypothesis that p38 MAPK and JNK are associated with cell death (Harper and LoGrasso, 2001). Although it was considered that both the ERK1/2 and PI3K/Akt signaling pathways were responsible for the CXCL1-induced Tau phosphorylation (Xia *et al*., 2002), and were therefore involved in the pathogenesis of AD, we provided evidence that inhibition of either ERK1/2 or PI3K/Akt pathway attenuated the MIP-2-induced neuroprotection. Out data suggest that the ERK1/2 and PI3K/Akt pathways are responsible for CXCR2-mediated neuronal survival.

Until the present study, the kinases downstream of ERK1/2 responsible for MIP-2-induced neuroprotection were not investigated in detail. We provided evidence for the first time that RSK, a family of serine/threonine kinases that lie at the terminus of the mitogen-regulated MEK-ERK pathway (Frodin and Gammeltoft, 1999), was phosphorylated and activated by MIP-2 treatment of the hippocampal neurons in a MEK-dependent manner. MIP-2 stimulation of the neuronal cells resulted in translocation of the RSK2 in nuclei, consistent with the previous report that CCL5 induced nuclear translocation of RSK in astrocytes (Zhang *et al*., 2002). Since inhibition of MEK1 by PD98059, which suppressed CXCR2-mediated RSK2 phosphorylation, significantly attenuated the receptor-mediated neuroprotection, it is conceivable that inhibition of RSKs may attenuate MIP-2 induced neuronal survival. As a matter of fact, previous studies have shown that inhibition of endogenous RSK function with specific antisense oligonucleotides blocked growth factor-dependent cell survival of several cell types, including fibroblasts and neurons (Bonni *et al*., 1999). RSK may be involved in neuroprotection by phosphorylating its substrates related to cell apoptosis and survival. Many of the RSK substrates, including Bad and CREB (Xing *et al*., 1996), are important

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for cell survival, suggesting the involvement of multiple signaling events down-stream of RSK in cell survival.

One of the important findings in this study is the MIP-2-induced phosphorylation of Bad. Bad is a proapoptotic member of the Bcl-2 family and is inactivated on phosphorylation via MAPK and PI3K pathways. Phosphorylation of Bad leads to the dissociation from pro-survival Bcl-2 proteins and the association of Bad with members of the 14-3-3 family of proteins. MIP-2 induced Bad phosphorylation at Ser-112 apparently involves the MEK1-ERK1/2-RSK pathway, whereas phosphorylation at Ser-136 apparently involves PI3K/Akt pathway. The importance of Bad phosphorylation in neuroprotection has been previously demonstrated (Datta *et al*. 1997). For instance, transforming growth factor-β1 increased Bad phosphorylation and protected neurons against damage (Zhu *et al*., 2002). The regulation of Bad by these phosphorylation events suggests that Bad is a point of convergence for multiple signaling pathways that cooperate in promoting cell survival.

CREB is a transcription factor expressed constitutively in neurons and is activated by phosphorylation at Ser133 residue. CREB plays a role in mediating adaptive responses of neurons to trans-synaptic stimuli (Yin *et al*., 1994; Yin *et al*., 1995). The present study showed that MIP-2 induced CREB phosphorylation in neuronal cells in a time-dependent manner. In addition, several other chemokines, including CX3CL1, CXCL-12, CCL5, and CCL22, have been reported to induce CREB phosphorylation in different cell types (Meucci *et al*., 1998; Zhang *et al*., 2002). The MIP-2-induced CREB phosphorylation is likely mediated by both the MEK1-ERK1/2 and PI3K-Akt pathways. Because the MEK1 and PI3K inhibitors reduced CXCR2-mediated

CREB phosphorylation as well as CXCR2-mediated neuroprotection, it is reasonably proposed that CREB phosphorylation may play an important role in MIP-2 induced neuroprotection. In addition, because β-amyloid (1-42) impairs activity-dependent CREB signaling in neurons (Tong *et al*., 2001), the MIP-2-induced activation of CREB appears to be particularly important for its protective effect on the neurotoxicity of β-amyloid. The importance of CREB in neuronal survival has also been demonstrated elsewhere. Mice deficient in CREB gene die perinatally before the majority of cerebellar granule neurons are generated (Rudolph *et al*., 1998). Studies on CREB null mutant embryos showed that CREB is necessary for the survival of peripheral neurons at the time of their neurotrophin dependency (Lonze *et al*., 2002). Analysis of the CREB-/- mouse embryos revealed a number of abnormalities in brain development that may reflect the contribution of CREB to the regulation of the survival of neurons (Bleckmann *et al*., 2002). Finally, recent findings link decreased CREB activity to neurotoxicity and neurodegeneration (Dawson and Ginty, 2002). Taken together, we demonstrate that MIP-2 protects hippocampal neurons against β-amyloid (1-42) neurotoxicity. The neuroprotective effect of MIP-2 involves the activation of MEK1-ERK1/2-RSK and PI3K/Akt signaling pathways, leading to the phosphorylation of RSKs, Bad and CREB.

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Footnotes

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Figure legends

Fig. 1. MIP-2 protected β-amyloid induced neuronal death. A, hippocampal neurons were treated with β-amyloid (1-42) in the presence of different concentrations of MIP-2 for 48 hours. Neuronal death was determined by TUNEL assay. The percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. B, hippocampal neurons were treated with β-amyloid (1-42) in the presence or absence of MIP-2 (10 nM) for different lengths of time. Neuronal death was determined by TUNEL assay. The percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. C, hippocampal neurons were incubated with a preimmune serum or a monoclonal CXCR2 antibody (1:100) for 60 min before being treated with β-amyloid (1-42) in the presence or absence of CXCL1 (10 nM) or CXCL8 for 40 hours. Neuronal death was determined by TUNEL assay. The percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. D, hippocampal neurons were treated with β-amyloid (1-42) in the presence or absence of CCL5 (10 nM) for different lengths of time. Neuronal death was determined by TUNEL assay. The percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. Data are mean ± S.E. from three independent experiments. *P < 0.05, ** P < 0.01, compared to the β-amyloid alone treated group. Aβ: β-amyloid (1-42).

Fig. 2. Involvement of ERK and Akt in MIP-2-induced neuprotection. A, hippocampal neuronal cultures were treated with MIP-2 (10 nM) for different lengths of time indicated. Phosphorylation of ERK1/2 and Akt was determined by Western blot analysis using anti-phospho-ERK1/2 and anti-phospho-Akt antibodies, respectively. The

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nitrocellulose membrane was stripped and reblotted with an anti-ERK2 antibody to confirm equal loading. Shown are representatives of three independent experiments with similar results. B, quantification of the density of bands representing phosphorylated ERK2 and Akt was determined by densitometric scanning from three independent experiments. C, hippocampal neuronal cultures were pre-treated with PD98059 or wortmannin for 60 min. Cells were incubated with β-amyloid (1-42) peptide (50 μM) in the presence or absence of MIP-2 (10 nM) for 48 hours. Neuronal death was determined by TUNEL assay. Percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. Data are mean ± S.E. from three independent experiments. *P < 0.05, compared to the control group treated with β-amyloid and MIP-2. Aβ, β-amyloid; PD, PD98059. D, hippocampal neuronal cultures were pre-treated with PD98059 or wortmannin for 60 min. Cells were incubated with β-amyloid (1-42) peptide (50 μM) in the presence or absence of CXCL8 (10 nM) for 48 hours. Neuronal death was determined by TUNEL assay. Percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. Data are mean ± S.E. from three independent experiments. *P < 0.05, compared to the control group treated with β-amyloid and CXCL8. E, hippocampal neuronal cultures were pre-treated with PD98059 or wortmannin for 60 min. Cells were incubated with β-amyloid (1-42) peptide (50 μM) in the presence or absence of CXCL1 (10 nM) for 48 hours. Neuronal death was determined by TUNEL assay. Percentage of TUNEL positive cells was calculated from 200 cells in 5 different fields. Data are mean ± S.E. from three independent

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experiments. *P < 0.05, compared to the control group treated with β-amyloid and CXCL1.

Fig. 3. MIP-2 induced RSK phosphorylation. A, hippocampal neuronal cultures were treated with MIP-2 (10 nM) for different lengths of time indicated. Phosphorylation of RSK1 and RSK2 was determined by Western blot analysis using specific antibodies against phospho-RSK1 and phospho-RSK2, respectively. The nitrocellulose membrane was stripped and reblotted with an anti-RSK1 antibody to confirm equal loading. Shown are representatives of three independent experiments with the similar results. B, quantification of the density of bands representing phosphorylated RSK2 was determined by densitometric scanning. Data are mean ± S.E. from three independent experiments. C, hippocampal neuronal cultures were pre-treated with PD98059 or wortmannin for 1 hour prior to the incubation with MIP-2 (10 nM) for 2 min. The phosphorylation of RSK2 was determined as described above. D. quantification of the density of bands representing phosphorylated RSK2 was determined by densitometric scanning from three independent experiments. Data were expressed as mean ± S.E. from three independent experiments. *P < 0.05, compared to the control group treated with MIP-2.

Fig. 4. MIP-2 induced nuclear translocation of phosphorylated RSK2. Hippocampal neurons were stimulated with or without MIP-2 (10 nM) for 1 or 5 min. Cells were incubated with an anti-phospho-RSK2 antibody for 30 min, followed by incubation with a Cy3-conjugated anti-mouse antibody in the presence of Hoechst 33258 for 30 min. The localization of the phosphorylated RSK2 was visualized under a fluorescent microscope. Shown are representatives of three independent experiments with the similar results.

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Fig. 5. MIP-2 induced Bad phosphorylation. A, hippocampal neuronal cultures were treated with MIP-2 (10 nM) for different lengths of time indicated. Phosphorylation of Bad at Ser-112 and Ser-136 was determined by Western blot analysis using monoclonal antibodies against phospho-Bad (Ser-112) and phospho-Bad (Ser-136), respectively. The nitrocellulose membrane was stripped and reblotted with an anti-Bad antibody to confirm equal loading. Shown are representatives of three independent experiments with the similar results. B, quantification of the density of bands representing phosphorylated Bad (Ser-112) and Bad (Ser-136) was determined by densitometric scanning. Data are mean ± S.E. from three independent experiments. C, hippocampal neuronal cultures were pre-treated with PD98059 or wortmannin for 1 hour prior to the incubation with MIP-2 (10 nM) for 5 min. The phosphorylation of Bad (Ser-112) and Bad (Ser-136) was determined as described above. Shown are representatives of three independent experiments with the similar results. D, B, quantification of the density of bands representing phosphorylated Bad (Ser-112) and Bad (Ser-136) was determined by densitometric scanning. Data are mean ± S.E. from three independent experiments from 200 cells in 5 different fields. *$P < 0.05$, compared to the control group treated with MIP-2.

Fig. 6. MIP-2 induced CREB phosphorylation. A, hippocampal neuronal cultures were treated with MIP-2 (10 nM) for different lengths of time indicated. Phosphorylation of CREB was determined by Western blot analysis using anti-phospho-CREB (Ser-133) antibody. The nitrocellulose membrane was stripped and reblotted with an anti-CREB antibody to confirm equal loading. Shown are representatives of three independent

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experiments with the similar results. B, quantification of the density of bands representing phosphorylated CREB was determined by densitometric scanning. Data are mean ± S.E. from three independent experiments. C, hippocampal neuronal cultures were pre-treated with PD98059 (100 nM) or wortmannin (100 nM) for 1 hour prior to the incubation with MIP-2 (10 nM) for 5 min. The phosphorylation of CREB was determined as described above. Shown are representatives of three independent experiments with the similar results. D, quantification of the density of bands representing phosphorylated CREB was determined by densitometric scanning. Data are mean ± S.E. from three independent experiments. *$P < 0.05$, compared to the control group treated with MIP-2.

Fig. 1

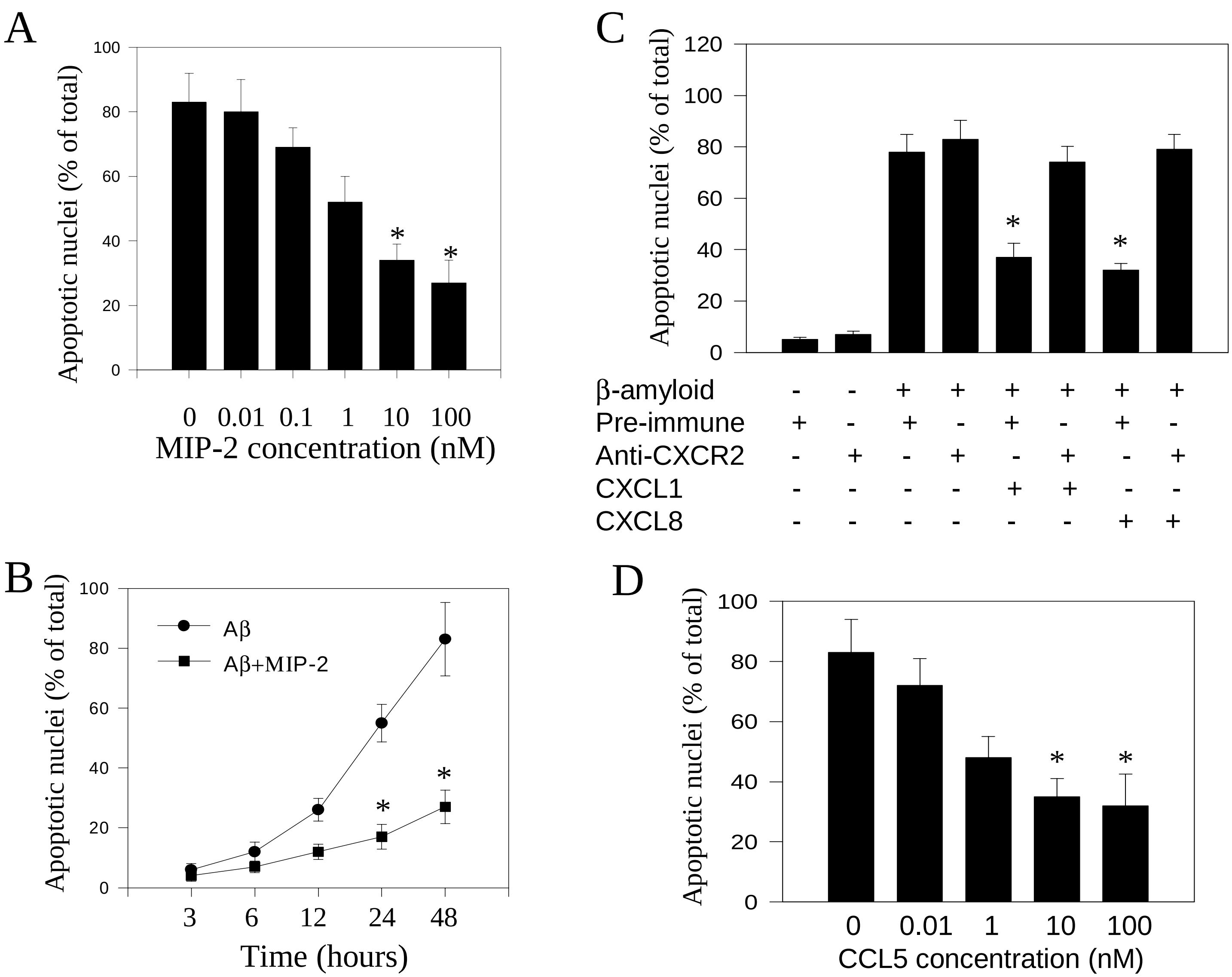

A
Apoptotic nuclei (% of total)
0
20
40
60
80
100
0 0.01 0.1 1 10 100
MIP-2 concentration (nM)
C
Apoptotic nuclei (% of total)
0
20
40
60
80
100
120
β-amyloid - - + + + + + +
Pre-immune + - + - + - + -
Anti-CXCR2 - + - + - + - +
CXCL1 - - - - + + - -
CXCL8 - - - - - - + +
B
Apoptotic nuclei (% of total)
Aβ
Aβ+MIP-2
3 6 12 24 48
Time (hours)
D
Apoptotic nuclei (% of total)
0 0.01 1 10 100
CCL5 concentration (nM)

Fig.2

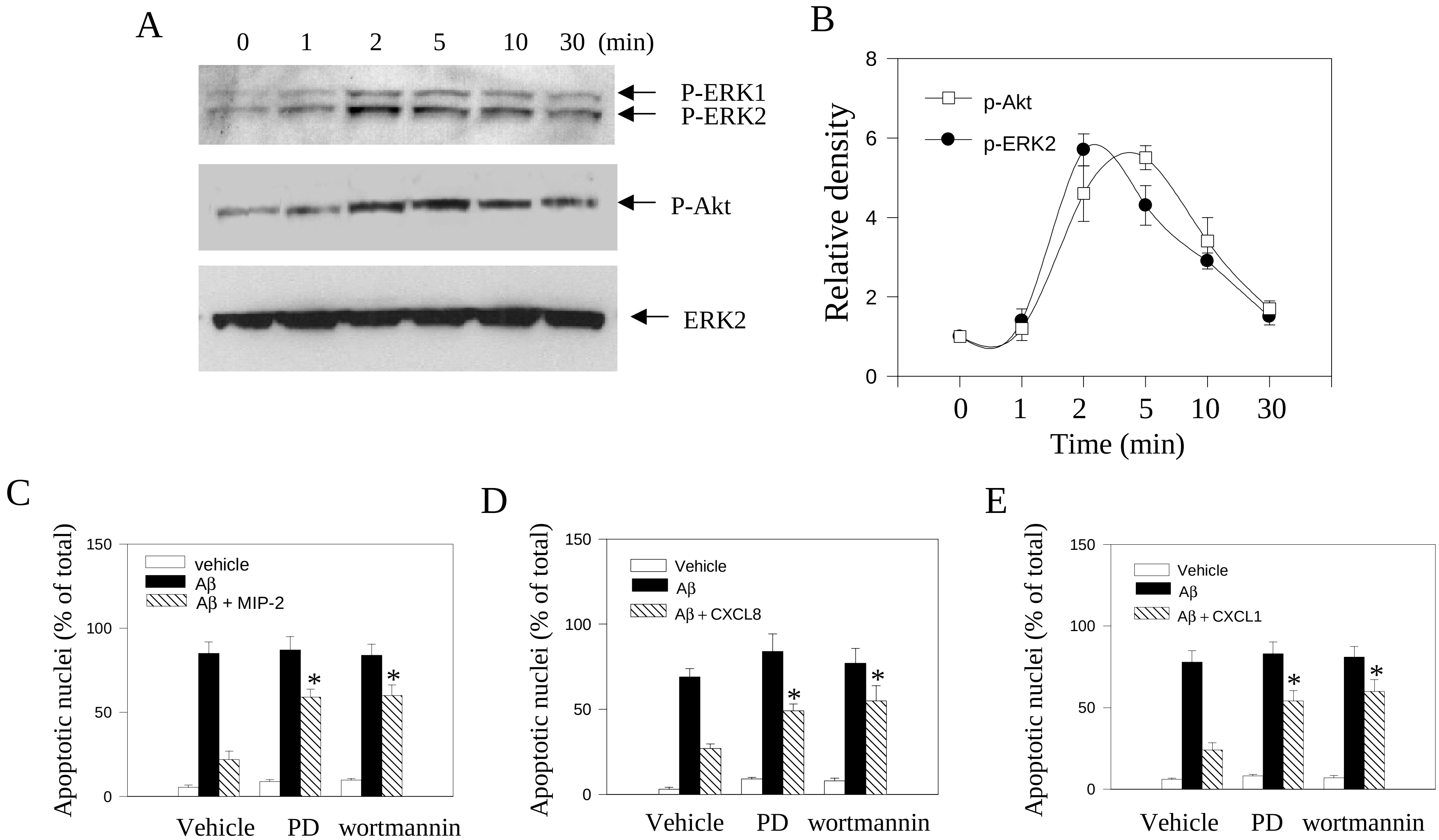

A
0 1 2 5 10 30 (min)
P-ERK1
P-ERK2
P-Akt
ERK2
B
Relative density
p-Akt
p-ERK2
Time (min)
C
Apoptotic nuclei (% of total)
vehicle
Aβ
Aβ + MIP-2
Vehicle
PD
wortmannin
D
Apoptotic nuclei (% of total)
Vehicle
Aβ
Aβ + CXCL8
Vehicle
PD
wortmannin
E
Apoptotic nuclei (% of total)
Vehicle
Aβ
Aβ + CXCL1
Vehicle
PD
wortmannin

Fig.3

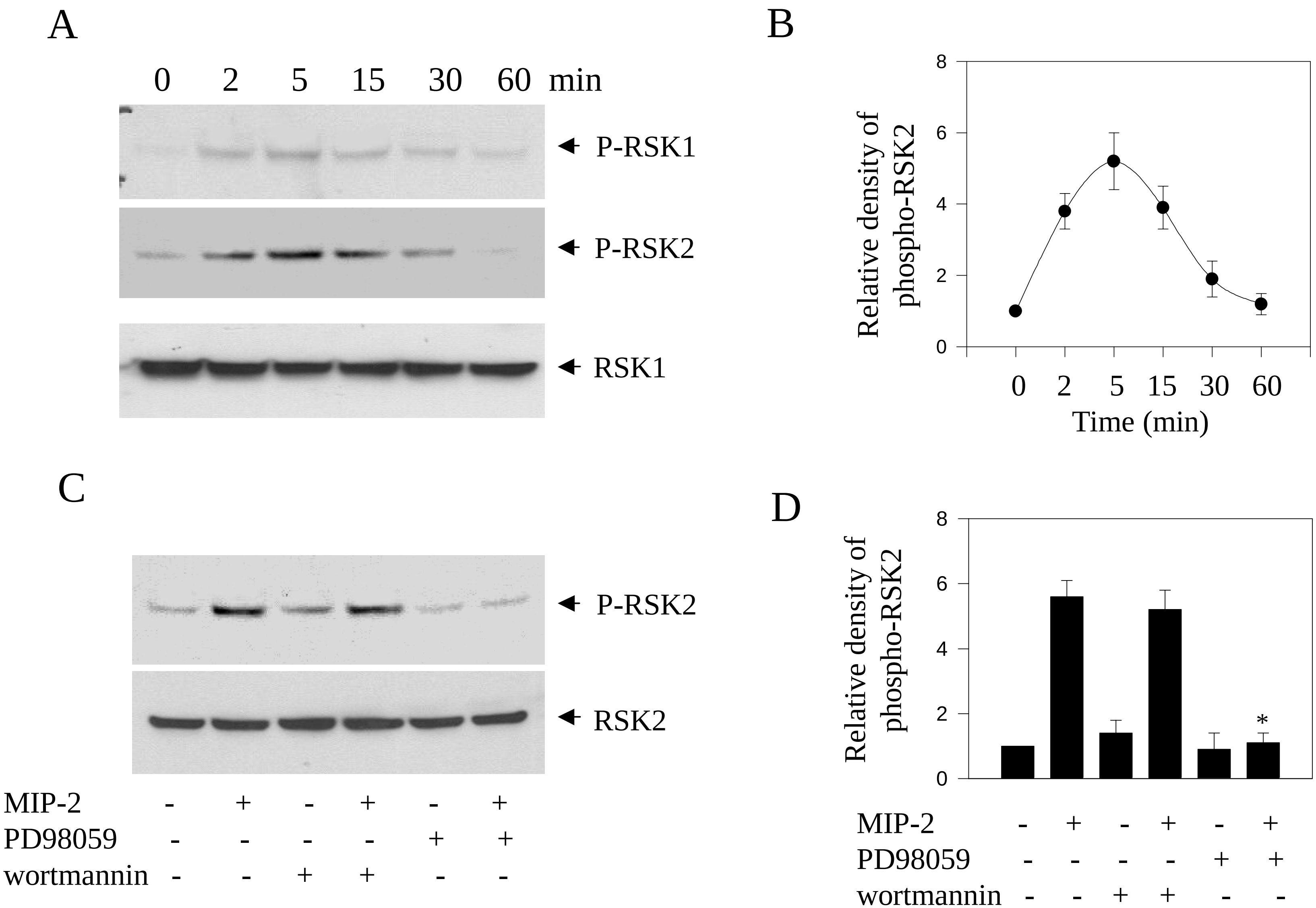

A
0 2 5 15 30 60 min
P-RSK1
P-RSK2
RSK1
B
Relative density of phospho-RSK2
0 2 4 6 8
0 2 5 15 30 60
Time (min)
C
P-RSK2
RSK2
MIP-2 - + - + - +
PD98059 - - - - + +
wortmannin - - + + - -
D
Relative density of phospho-RSK2
0 2 4 6 8
*
MIP-2 - + - + - +
PD98059 - - - - + +
wortmannin - - + + - -

Fig. 4.

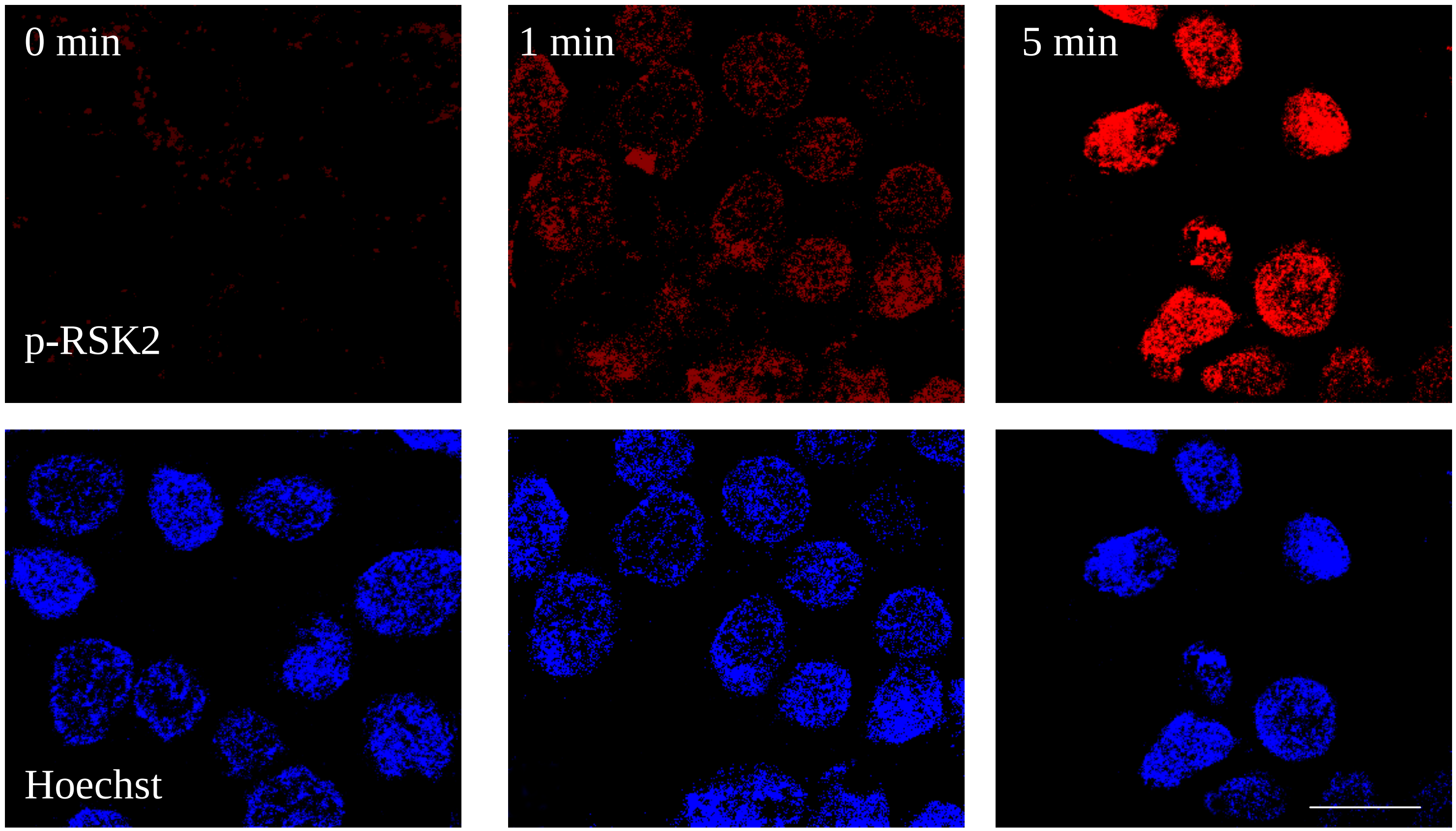


Fig.5

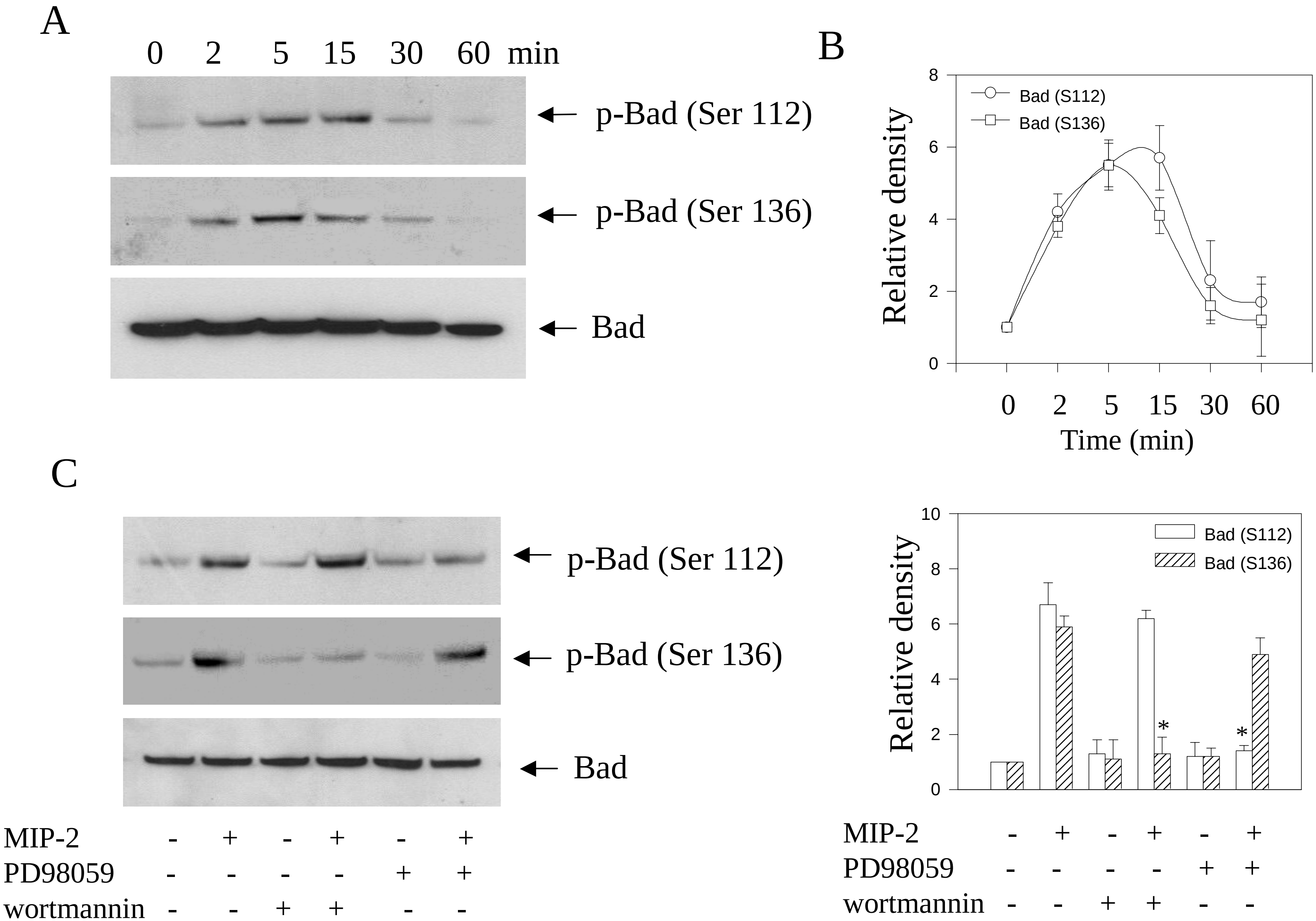
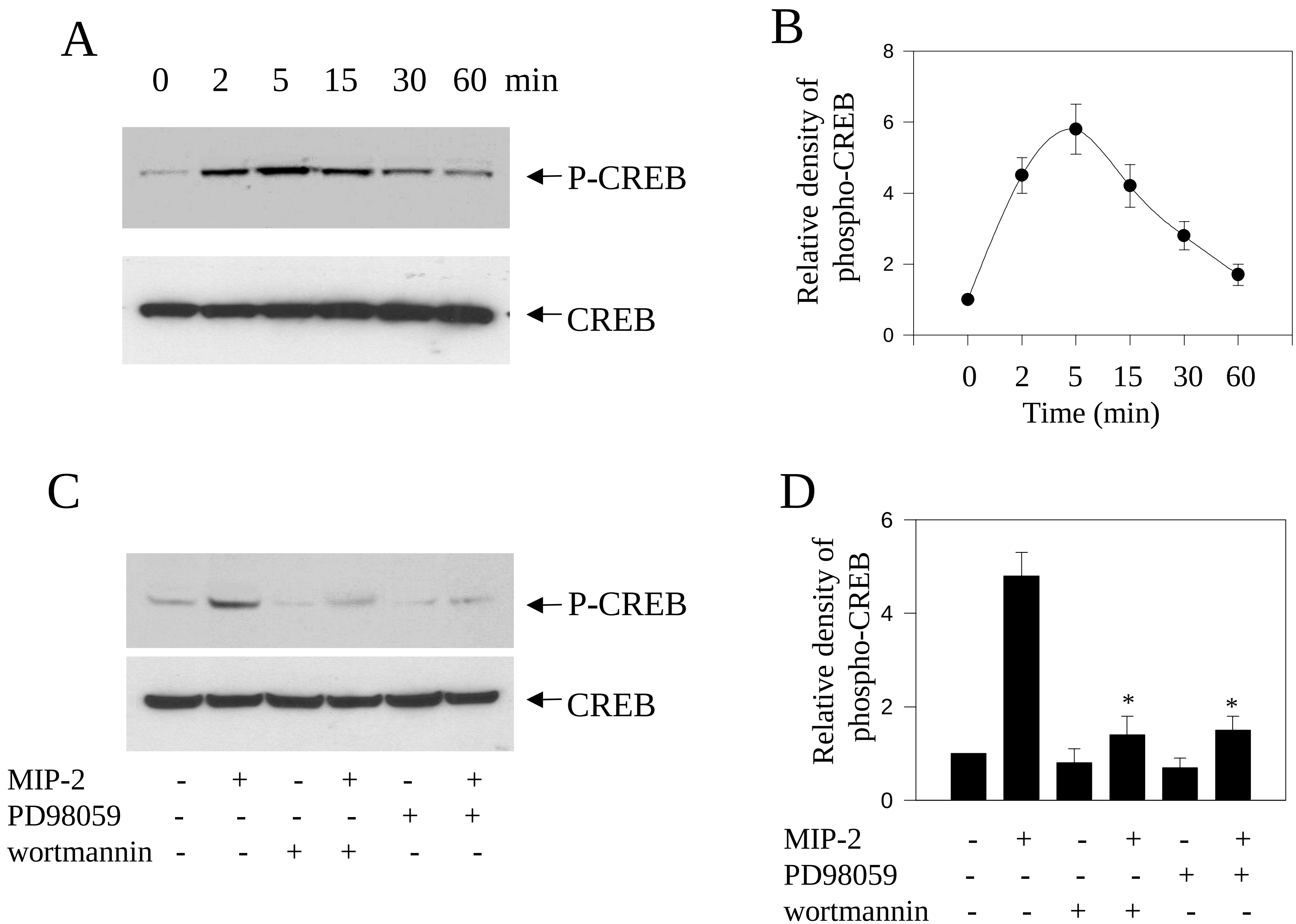

A
0 2 5 15 30 60 min
p-Bad (Ser 112)
p-Bad (Ser 136)
Bad
B
Bad (S112)
Bad (S136)
Relative density
8
6
4
2
0
0 2 5 15 30 60
Time (min)
C
p-Bad (Ser 112)
p-Bad (Ser 136)
Bad
MIP-2 - + - + - +
PD98059 - - - - + +
wortmannin - - + + - -
Bad (S112)
Bad (S136)
Relative density
10
8
6
4
2
0
*
*
MIP-2 - + - + - +
PD98059 - - - - + +
wortmannin - - + + - -

Fig. 6



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