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# $Zn^{2+}$ Induces Stimulation of the c-Jun N-Terminal Kinase Signaling Pathway through Phosphoinositide 3-Kinase

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## ABSTRACT

$Zn^{2+}$, one of the most abundant trace metal ions in mammalian cells, modulates the functions of many regulatory proteins associated with a variety of cellular activities. In the central nervous system, $Zn^{2+}$ is highly localized in the cerebral cortex and hippocampus. It has been proposed to play a role in normal brain function as well as in the pathophysiology of certain neurodegenerative disorders. We here report that $Zn^{2+}$ induced stimulation of the c-Jun N-terminal kinase (JNK) pathway in mouse primary cortical cells and in various cell lines. Exposure of cells to $Zn^{2+}$ resulted in the stimulation of JNK and its upstream kinases including stress-activated protein kinase kinase and mitogen-activated protein kinase kinase kinase. $Zn^{2+}$ also induced stimulation of phosphoinositide 3-kinase (PI3K) The $Zn^{2+}$-induced JNK stimulation was blocked by LY294002, a PI3K inhibitor, or by a dominant-negative mutant of PI3K$\gamma$. Furthermore, overexpression of Rac1N17, a dominant negative mutant of Rac1, suppressed the $Zn^{2+}$- and PI3K$\gamma$-induced JNK stimulation. The stimulatory effect of $Zn^{2+}$ on both PI3K and JNK was repressed by the free-radical scavenging agent *N*-acetylcysteine. Taken together, our data suggest that $Zn^{2+}$ induces stimulation of the JNK signaling pathway through PI3K-Rac1 signals and that the free-radical generation may be an important step in the $Zn^{2+}$ induction of the JNK stimulation.

$Zn^{2+}$ is an abundant trace metal ion in the human body and an essential component of many metalloproteins such as metalloenzymes, zinc-finger transcription factors, RING finger proteins, or metallothionein proteins (Choi and Koh, 1998). In the central nervous system, $Zn^{2+}$ is highly localized to synaptic boutons (Perez-Clausell and Danscher, 1985), where synaptic $Zn^{2+}$ can be released in concentrations of up to several hundred micromolar during synaptic activity (Assaf and Chung, 1984). Once released, $Zn^{2+}$ can modulate synaptic transmission by inhibiting *N*-methyl-D-aspartate currents, potentiating $\alpha$-amino-hydroxy-5-methyl-4-isoxazol propionic acid/kainate currents, or antagonizing $\gamma$-aminobutyric acid-mediated inhibitory responses (Westbrook and Mayer, 1987; Forsythe et al., 1988; Rassendren et al., 1990). In addition, $Zn^{2+}$ enters into neurons through *N*-methyl-D-aspartate receptors or voltage-gated $Ca^{2+}$ channels, leading to neuronal death through mechanisms involving the production of free radicals (Choi and Koh, 1998; Kim et al., 1999b). This suggests that $Zn^{2+}$ acts as a key modulator of neuronal activity and death. However, the signaling pathways underlying physiological and cytotoxic actions of $Zn^{2+}$ remain to be resolved.

The mitogen-activated protein kinase (MAPK) pathway typically mediates intracellular signals initiated by extracellular stimuli to the nucleus. The MAPK signaling cascade participates in regulating a variety of cellular activities such as cell growth, differentiation, survival, or death (Seger and Krebs, 1995; Xia et al., 1995). The MAPK signaling pathway consists of three components of the protein kinase family: MAPKs, MAPK kinases, and MAPK kinase kinases. The mammalian MAPKs include three distinct subfamilies: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK), and p38 (Cano and Mahadevan, 1995; Minden and Karin, 1997; Ip and Davis, 1998). The ERK pathway, which is often stimulated by mitogens such as peptide growth factors, is composed of ERK and upstream kinases, including MEK1 and Raf-1. The p38 pathway, which can be stimulated by various stresses, including osmotic stress, consists of p38 and upstream kinases, including MKK3, MKK4, or MKK6. Like the p38 pathway, the JNK/SAPK pathway can be also stimulated

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**ABBREVIATIONS:** MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; PI3K, phosphoinositide 3-kinase; AP-1, activator protein 1; ATF-2, activating transcription factor 2; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; SEK, stress-activated protein kinase kinase; MEKK, mitogen-activated protein kinase kinase kinase.

by a variety of cellular stresses, including DNA damage, free radicals, heat shock, osmotic shock, or proinflammatory cytokines such as tumor necrosis factor-$\alpha$ and interleukin-1$\beta$ (Cano and Mahadevan, 1995; Minden and Karin, 1997; Ip and Davis, 1998). The JNK/SAPK pathway consists of JNK/SAPK plus upstream kinases, including SEK1/JNKK1/MKK4 and MEKK1. It has recently been reported that signal transmission from extracellular stimuli to the MEKK1-SEK1-JNK pathway can be controlled by several upstream regulators, including phosphoinositide 3-kinase (PI3K), and the small GTP-binding proteins Rac1 and Cdc42 (Coso et al., 1996; Voyno-Yasenetskaya et al., 1996). JNK/SAPK phosphorylates c-Jun, which is a major component of the transcription factor AP-1 complex and other transcription factors such as ATF-2 and Elk1 (Derijard et al., 1994; Gupta et al., 1995; Whitmarsh et al., 1995). Thus, JNK/SAPK can stimulate AP-1 through the c-Jun phosphorylation (Ip and Davis, 1998). The physiological function of the JNK/SAPK is not yet fully understood, but it has been implicated in stress-activated signaling processes (Minden and Karin, 1997; Ip and Davis, 1998).

In the present study, we investigated possible effects of $Zn^{2+}$ on the MAPK signaling pathway in primary mouse cortical cells and HNN8 neuroblastoma cells. We report that $Zn^{2+}$ stimulates JNK/SAPK activity through the MEKK1-SEK1-JNK signaling cascade. Moreover, our study suggests that PI3K$\gamma$ and Rac1 are involved in the $Zn^{2+}$-induced stimulation of the JNK/SAPK pathway. The elucidation of the $Zn^{2+}$-induced JNK/SAPK activation, therefore, may be important to the understanding of the mechanism by which zinc modulates the intracellular signaling cascades involved in a variety of brain functions.

## Materials and Methods

**Cell Culture and Transfection.** Primary mouse cortical cultures were prepared as described previously (Ko et al., 1998). Briefly, neocortical cells were prepared from a 15-day-old mouse embryo and plated in six-well plates ($2 \times 10^6$ cells/plate) precoated with 100 $\mu$g/ml poly-D-lysine and 4 $\mu$g/ml laminin. Neuron-rich cortical cell cultures were prepared by cultivating cortical cells in Dulbecco's modified Eagle's medium supplemented with 10 $\mu$M cytosine arabinoside, 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose in a humidified 5% $CO_2$ incubator. C6 glioma cells, BV2 microglial cells, and HNN8 cells, which are hybrid cells between sy5y-AG neuroblastoma cells and mouse E12 cortical neurons, were cultivated at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% $CO_2$ incubator. Cells were transfected with appropriate plasmid vectors by using LipofectAMINE (Life Technologies, Inc., Rockville, MD).

**Immunocomplex Kinase Assays.** Cells were lysed with buffer A containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Solubilized fractions were subjected to immunoprecipitation with appropriate antibodies that included mouse monoclonal anti-JNK1 (PharMingen, San Diego, CA), mouse monoclonal anti-p38 (PharMingen), rabbit polyclonal anti-ERK2 (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal anti-SEK1 (PharMingen), rabbit polyclonal anti-MEKK1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-HA (Roche Molecular Biochemicals, Mannheim, Germany), or mouse monoclonal anti-FLAG (Stratagene, La Jolla, CA) antibody, respectively. Immunocomplex kinase assays were performed by incubating the immunopellets for 30 min at 30°C with 2 $\mu$g of indicated substrate proteins in 20 $\mu$l of the reaction buffer containing 0.2 mM sodium orthovanadate, 2 mM dithiothreitol, 10 mM $MgCl_2$, 1 $\mu$Ci of [$\gamma^{32}$P]ATP, and 20 mM HEPES, pH 7.4. The reaction was terminated by adding 5 $\mu$l of 5× sample buffer and boiling the solution for 3 min. The reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel. The phosphorylated substrates were visualized using a Fuji BAS 2500 image analyzer (Fujifilm, Tokyo, Japan). GST-ATF2 was used as a substrate for JNK and p38, GST-SAPK$\beta$ for SEK1, GST-SEK1 for MEKK1, or myelin basic protein for ERK2. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA).

**Phosphoinositide 3-Kinase Assay.** PI3K activity was measured as described previously (Soltoff et al., 1992). HNN8 cells were treated with indicated agents, washed twice with a cold washing buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM $CaCl_2$), and then lysed with a cold lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 200 $\mu$M vanadate). Cell lysates (600 $\mu$g of protein) were subjected to immunoprecipitation using 5 $\mu$g of mouse monoclonal antiphosphotyrosine antibody (4G10; Upstate Biotechnology) and protein A-Sepharose beads (5 mg/$\mu$g antibody). The immunopellets were washed with buffer I (137 mM NaCl, 15.7 mM $NaH_2PO_4$, 1.47 mM $KH_2PO_4$, 2.68 mM KCl, 1% Nonidet P-40, and 200 $\mu$M vanadate), buffer II (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, and 200 $\mu$M vanadate), and buffer III (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 200 $\mu$M vanadate). The immunopellets were then incubated for 10 min at 25°C in a reaction mixture containing 20 mM $MgCl_2$, 0.5 mg/ml sonicated phosphatidylinositol, 60 $\mu$M ATP, 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP in buffer III. The reaction was terminated by the addition of 20 $\mu$l HCl (8 M) and 160 $\mu$l of methanol/chloroform (1:1). The lower organic phase was recovered and spotted on 1% oxalate-coated silica gel thin-layer chromatography plate. After being developed in chloroform/methanol/water/ammonium hydroxide (120:94:23.2:4) for 30 to 60 min, the plate was exposed on an X-ray film.

**Luciferase Reporter Assay for c-Jun-dependent Transcription.** The transcription stimulating activity of c-Jun was measured using the PathDetect luciferase reporter kit (Stratagene). HNN8 cells were transiently transfected with appropriate plasmids (pFR-Luc, pFA2-c-Jun, or pFC2-dbd; Stratagene) as indicated. PSV-$\beta$-gal was also included in all transfections. Cell lysates from transfected cells were subjected to microcentrifugation at 4°C for 10 min. The resultant soluble fraction was analyzed for luciferase activity using a luciferase assay kit (Promega, Madison, WI). The luciferase activity was normalized with reference to the $\beta$-galactosidase activity in each sample.

**Measurement of Reactive Oxygen Species (ROS) Generation.** Cells were exposed to the indicated reagents and were incubated for 15 min at 37°C under 10% $CO_2$ in Krebs-Ringer's solution containing 1 $\mu$g/ml 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR). The cells were then washed twice with Krebs-Ringer's solution and incubated for 5 min at room temperature with dimethyl sulfoxide. The intensity of fluorescence was measured at an excitation wavelength of 485 nm, and an emission wavelength of 530 nm using BioAssay Reader (HTS 7000; PerkinElmer, Emeryville, CA).

## Results

**$Zn^{2+}$ Induces the Stimulation of the MEKK1-SEK1-JNK Signaling Cascade.** To assess possible effects of $Zn^{2+}$ on the MAPK signaling cascades, we examined the effect of zinc chloride on JNK1, p38, and ERK2 in mouse cortical cell culture. Exposure of cortical cell culture (DIV 12–14) to 100 $\mu$M zinc chloride resulted in a strong enhancement of JNK1 activity (Fig. 1A). Both p38 and ERK2 activities were also elevated upon exposure of the cells to zinc chloride. Our data, therefore, indicate that $Zn^{2+}$ can modulate the MAPK signaling pathways in mouse cortical cells. $Zn^{2+}$ also induced JNK1 activation in other cell types including C6 glioma cells, BV2 microglial cells, and HNN8 neuroblastoma cells (Fig. 1,

B and C). Immunoblot analysis using anti-phospho JNK antibody also indicated that the $Zn^{2+}$ treatment enhanced the phosphorylation of intracellular JNK1 in HNN8 cells (Fig. 1D). The relative fold stimulation of JNK1 activity by 200 $\mu$M $Zn^{2+}$ was comparable with those of 10 $\mu$g/ml anisomycin or 60 J/m$^2$ UV light (Fig. 1E). We then investigated the mechanism by which $Zn^{2+}$ induced the stimulation of the JNK signaling pathway in the following experiments.

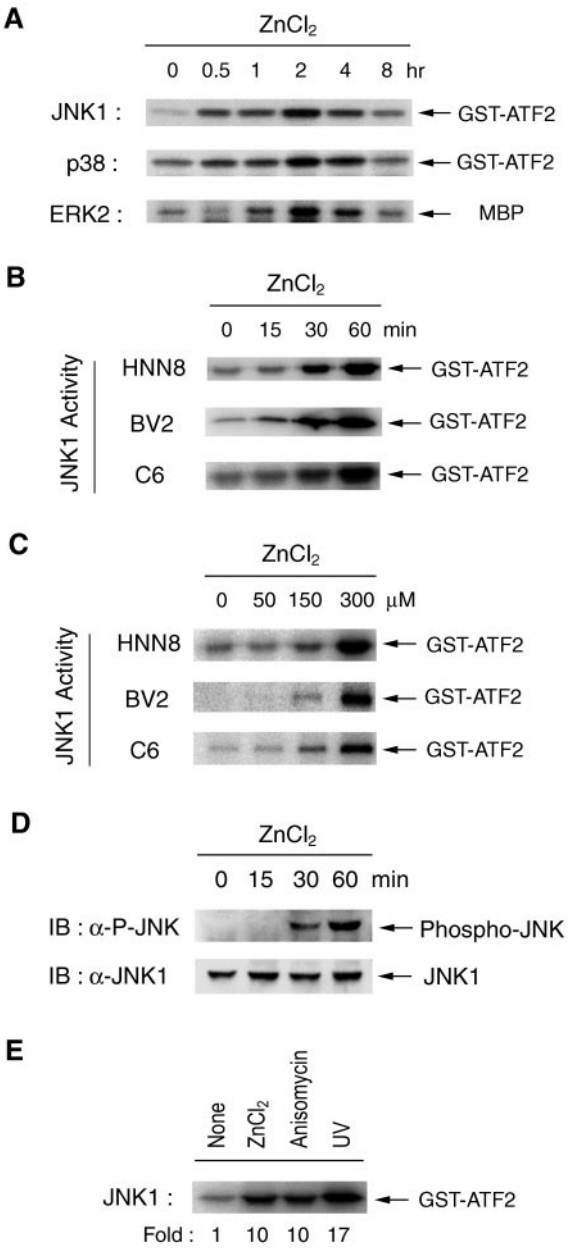


**Fig. 1.** Zinc chloride induces stimulation of MAPKs in intact cells. A, primary mouse brain cortical cells in culture were exposed to 100 $\mu$M zinc chloride for 30 min and incubated further for the indicated time periods. Cell lysates were subjected to immunoprecipitation with anti-JNK1, anti-p38, or anti-ERK2 antibody, respectively, and analyzed for protein kinase activity by immunocomplex kinase assay using GST-ATF2 or myelin basic protein (MBP) as substrate. B and C, HNN8, BV2, or C6 cells were exposed to 200 $\mu$M $ZnCl_2$ for the indicated time periods (B) or exposed to indicated concentrations of $ZnCl_2$ for 1 h (C). D, HNN8 cells were treated with 200 $\mu$M $ZnCl_2$ for indicated times. The cell lysates were subjected to immunoprecipitation with anti-JNK1 antibody. The immunopellets were subjected to SDS-polyacrylamide gel electrophoresis and then analyzed by immunoblotting probed with anti-phospho JNK antibody (Santa Cruz Biotechnology) or anti-JNK1 antibody. E, HNN8 cells were treated with either 200 $\mu$M $ZnCl_2$ or 10 $\mu$g/ml anisomycin for 1 h, or treated with 60 J/m$^2$ UV light and then further incubated for 1 h. Fold increase in phosphorylation of GST-ATF2 is indicated. In B, C, and E, cell lysates were subjected to immunoprecipitation with anti-JNK1 antibody, and the immunopellets were assayed for JNK1 activity as in A.

The JNK signaling cascade is composed of JNK and its upstream kinases, which include SEK1 and MEKK1. We tested whether $Zn^{2+}$ could induce stimulation of the upstream kinases SEK1 and MEKK1 in HNN8 cells (Fig. 2). Exposure of the cells to 200 $\mu$M zinc chloride resulted in an increase in SEK1 activity (Fig. 2A) as well as MEKK1 activity in HNN8 cells (Fig. 2B). The data, therefore, suggest that $Zn^{2+}$ induces stimulation of the MEKK1-SEK1-JNK signaling cascade.

**$Zn^{2+}$ Activates the JNK Pathway via Phosphoinositide 3-Kinase.** One of the intracellular regulators upstream of the JNK pathway is PI3K (Lopez-Ilasaca et al., 1998). We, therefore, tested whether PI3K was involved in a mechanism underlying the $Zn^{2+}$-induced stimulation of the JNK signaling pathway. First, we looked for an effect of $Zn^{2+}$ on the PI3K activity in HNN8 cells, and we found that exposure of the cells to $Zn^{2+}$ enhanced the PI3K activity in a time-dependent manner (Fig. 3A). Next, we examined whether LY294002, a PI3K inhibitor, would mitigate the stimulatory effect of $Zn^{2+}$ on JNK1 activity (Fig. 3B). Our data indicate that pretreatment of HNN8 cells to LY294002 resulted in a decrease in the $Zn^{2+}$-stimulated JNK1 activity. LY294002 also inhibited the $Zn^{2+}$-induced stimulation of MEKK1 activity. We then further examined the role of PI3K in the $Zn^{2+}$-induced stimulation of the JNK pathway by using a dominant-negative mutant of PI3K$\gamma$, PI3K$\gamma$-DN, in which an arginine residue is substituted for a lysine residue at amino acid position 832 (Fig. 3C). HNN8 cells were transfected with plasmid vectors expressing JNK1 and PI3K$\gamma$-DN. The ectopic expression of PI3K$\gamma$-DN indeed suppressed the $Zn^{2+}$-induced stimulation of JNK1 activity in the transfected cells. Similarly, the $Zn^{2+}$-induced stimulation of MEKK1 was also suppressed in these cells. The data, therefore, suggest that PI3K$\gamma$ may be involved in the mechanism by which $Zn^{2+}$ induces stimulation of the JNK signaling pathway.

There are many lines of evidence that the small GTPase Rac1 may act downstream of PI3K under various conditions (Rodriguez-Viciana et al., 1997; Missy et al., 1998; Kim et al., 1999a). To test whether Rac1 is involved in the $Zn^{2+}$-induced

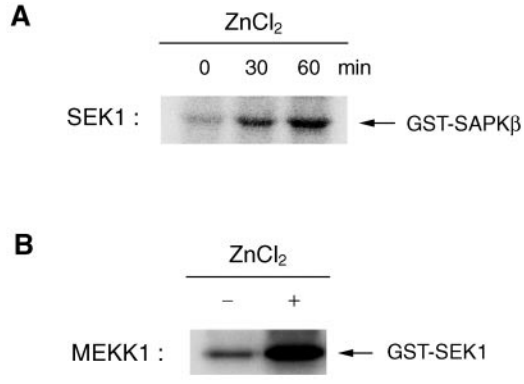


**Fig. 2.** Zinc chloride induces stimulation of SEK1 or MEKK1 activity in HNN8 neuroblastoma cells. HNN8 cells were exposed to 200 $\mu$M $ZnCl_2$ for the indicated time periods (A) or 30 min (B). Cell lysates were then subjected to immunoprecipitation with anti-SEK1 (A) or anti-MEKK1 (B) antibody. The immunopellets were assayed for either SEK1 (A) or MEKK1 (B) activity using either GST-SAPK$\beta$(K55R) or GST-SEK1 as substrate.

JNK stimulation, we transfected HNN8 cells with plasmids expressing JNK1 and Rac1N17 (Fig. 4A). Rac1N17 is a dominant negative mutant of Rac1. Our data indicate that the $Zn^{2+}$-induced stimulation of the JNK1 activity was suppressed in the Rac1N17-transfected cells. Overexpressed Rac1N17 also blocked the activation of JNK1 induced by PI3K$\gamma$-CAAX, a membrane-targeted form of PI3K$\gamma$ (Fig. 4B). Collectively, our results suggest that overexpression of Rac1N17 suppressed the $Zn^{2+}$- and PI3K$\gamma$-induced JNK stimulation.

**$Zn^{2+}$ Stimulates c-Jun-Dependent Luciferase Reporter Expression.** C-Jun is one of the primary targets of JNK (Ip and Davis, 1998). When activated, JNK can phosphorylate c-Jun and its phosphorylation results in the activation of the transcription stimulating activity of c-Jun (Ip and Davis, 1998). We therefore examined the effect of $Zn^{2+}$ on the transcriptional activity of c-Jun by luciferase reporter assay (Fig. 5). Our results indicate that $Zn^{2+}$ induced an enhancement of the *trans*-activation activity of c-Jun in primary mouse cortical cells (Fig. 5A) and in HNN8 cells (Fig. 5B).

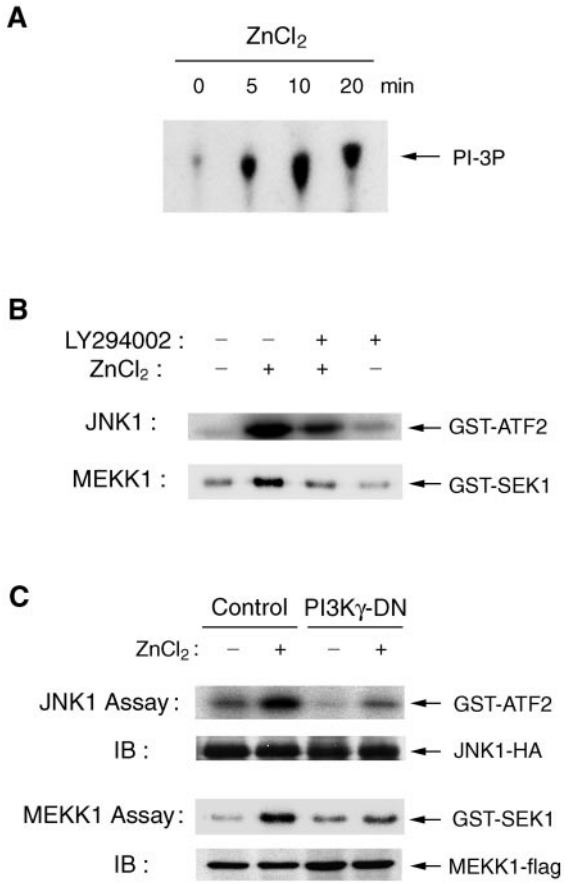


**Fig. 3.** Phosphoinositide 3-kinase is involved in the $Zn^{2+}$-induced JNK activation. A, HNN8 cells were exposed to 200 $\mu$M $ZnCl_2$ for the indicated time periods, and the cell lysates were assayed for PI3K activity as described under *Materials and methods*. B, HNN8 cells were pretreated without or with 100 $\mu$M LY294002 for 30 min and then treated with 200 $\mu$M $ZnCl_2$ for 1 h. Cell lysates were then subjected to immunoprecipitation, and the resultant immunopellets were assayed for JNK1 or MEKK1 activity by immunocomplex kinase assay. C, HNN8 cells were cotransfected with plasmids expressing JNK1-HA or MEKK1-flag and PI3K$\gamma$-DN, as indicated. After 48 h of transfection, the cells were exposed to 200 $\mu$M $ZnCl_2$ for 1 h. Cells were then harvested, lysed, immunoprecipitated with anti-HA or anti-flag antibody, and examined for JNK1 or MEKK1 activity by immunocomplex assay. Cell lysates were also subjected to immunoblotting (IB) with anti-HA or anti-flag antibody using an enhanced chemiluminescence system. DN, a dominant-negative mutant.

***N*-Acetylcysteine Can Block $Zn^{2+}$-Induced JNK Stimulation.** It has been shown previously that exposure of cells to $Zn^{2+}$ can enhance the intracellular level of ROS (Kim et al., 1999b). ROS is a potent activator of the JNK signaling pathway (Lo et al., 1996). We therefore examined whether ROS generation might be involved in the mechanism of the $Zn^{2+}$-induced JNK stimulation. Exposure of HNN8 cells to 200 $\mu$M zinc chloride produced increased ROS generation, and the $Zn^{2+}$-induced ROS generation was suppressed by pretreatment of the cells with the free-radical scavenger *N*-acetylcysteine (NAC) (Fig. 6A). NAC also blocked the $Zn^{2+}$-induced stimulation of PI3K activity (Fig. 6B). These results suggest that ROS mediates the $Zn^{2+}$-induced stimulation of PI3K activity. Indeed, PI3K activity was also stimulated by $H_2O_2$, another agent that induces ROS generation (Fig. 6B). Moreover, NAC suppressed the $Zn^{2+}$-induced stimulation of JNK activity (Fig. 6C) and the *trans*-activating activity of c-Jun (Fig. 6D). Taken together, our data suggest that $Zn^{2+}$ induces stimulation of the JNK signaling pathway through ROS generation.

## Discussion

In the central nervous system, $Zn^{2+}$ is highly present in nerve terminal boutons (Frederickson, 1989). It has been shown that zinc in presynaptic nerve terminals can be released in concentrations of up to several hundred micromolar during synaptic activity (Assaf and Chung, 1984) and that this may modulate the functions of several ion channels and cell surface receptors for different neurotransmitters including excitatory and inhibitory ones (Choi and Koh, 1998). In this context, $Zn^{2+}$ has been shown to inhibit the function of

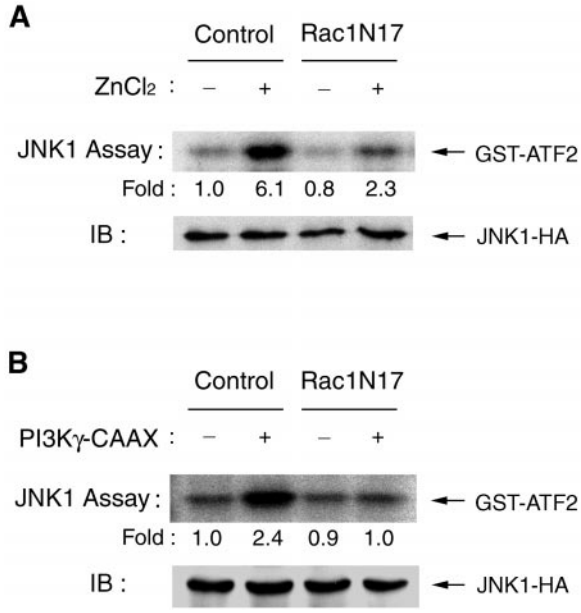


**Fig. 4.** Zinc-induced JNK stimulation is blocked by overexpressed Rac1N17. HNN8 cells were cotransfected with plasmid vectors expressing JNK1-HA, Rac1N17, or PI3K$\gamma$-CAAX as indicated. In A, the transfected cells were treated with 200 $\mu$M $ZnCl_2$ for 1 h after 48 h of transfection. In A and B, the transfected cells were subjected to immunoprecipitation using anti-HA antibody and the resultant immunopellets were examined for JNK1 activity by immunocomplex kinase assay. Cell lysates were also analyzed by immunoblot (IB) probed with anti-HA antibody. Fold increase in phosphorylation of a substrate protein is indicated.

*N*-methyl-D-aspartate receptor (Christine and Choi, 1990; Ascher, 1998) but to facilitate α-amino-hydroxy-5-methyl-4-isoxazol propionic acid receptor-mediated neuronal activity (Rassendren et al., 1990). In addition, zinc can enter cells using its transport proteins and exert interactions with a number of intracellular proteins, including the regulatory proteins involved in signal transduction. For instance, zinc has been shown to modulate the functions of protein kinase C (Hedberg et al., 1994), calmodulin (Baudier et al., 1983), caspase-3 (Perry et al., 1997), and nuclear factor-κB (Shumilla et al., 1998). The elucidation of the action of zinc on intracellular signaling is, therefore, critical to the understanding of zinc's regulatory role in normal brain function as well as in the pathophysiology of neurological diseases.

In the present study, we showed that $Zn^{2+}$ induces stimulation of JNK and other MAP kinases in primary mouse cortical cells and several established cell lines. Moreover, our data suggest that $Zn^{2+}$ induces the JNK stimulation through the MEKK1-SEK1-JNK signaling cascade, and that PI3K and Rac1 may mediate the $Zn^{2+}$-induced stimulation of the MEKK1-SEK1-JNK signaling pathway. The PI3K family has several isoforms, including α, β, δ, and γ forms (Vanhaesebroeck and Waterfield, 1999). The α, β, or δ form of PI3K is composed of a p110 catalytic subunit and a p85 adapter

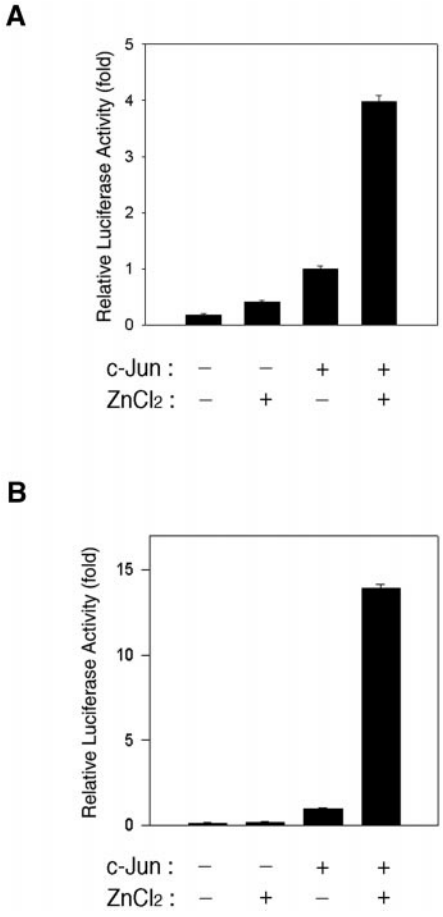


**Fig. 5.** $Zn^{2+}$ induces c-Jun-mediated luciferase reporter gene activity. Primary mouse cortical cells (A) or HNN8 cells (B) were cotransfected with appropriate plasmids (pFR-Luc, pFA2-c-Jun, or pFC2 empty vector) along with pSV-β-gal. At 24 h after transfection, Cells were exposed to 100 μM $ZnCl_2$ for 2 h, incubated further for 8 h, and then assayed for luciferase activity. Luciferase activity in each sample was normalized according to the β-galactosidase activity measured.

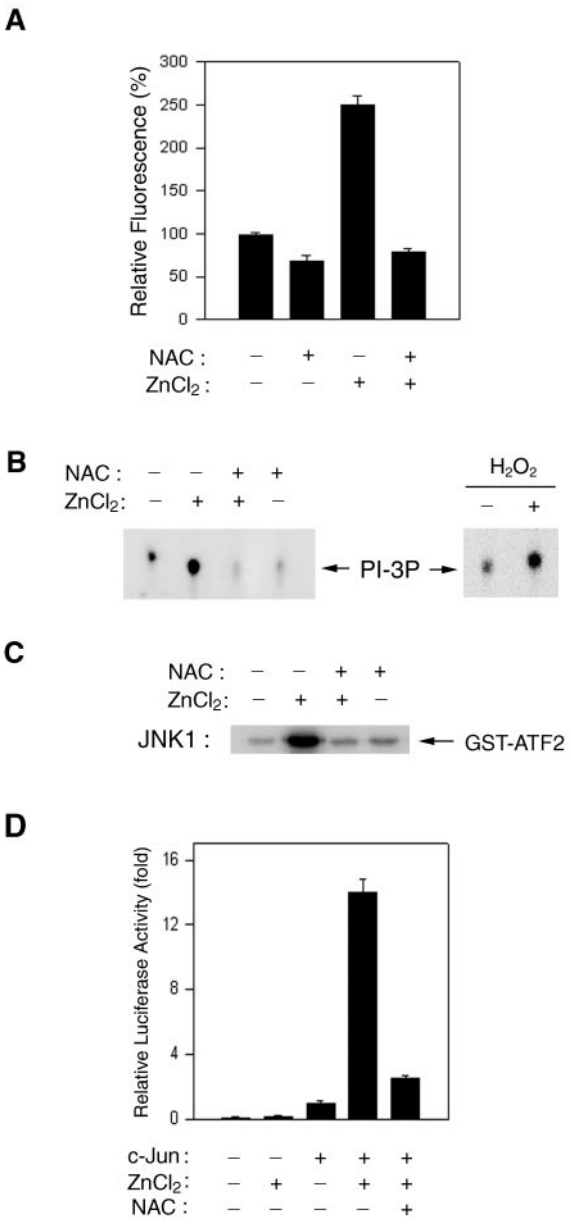


**Fig. 6.** *N*-acetylcysteine prevents $Zn^{2+}$ from inducing JNK stimulation. A, *N*-acetylcysteine (NAC) blocks the zinc-induced generation of ROS. HNN8 cells were pretreated with 30 mM *N*-acetylcysteine for 1 h, and then exposed to 200 μM $ZnCl_2$ for 30 min. After washing with Krebs-Ringer's solution, the cells were incubated for 15 min at 37°C in Krebs-Ringer's solution containing 1 μg/ml 2′,7′-dichlorofluorescin diacetate. Cells were then washed and incubated for 5 min at room temperature with dimethyl sulfoxide. The intensity of fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a spectrofluorometer. Fluorescence intensity values are shown as percentages of initial values after the background value were subtracted. B, *N*-acetylcysteine blocks $Zn^{2+}$-induced PI3K stimulation. HNN8 cells were pretreated with 30 mM *N*-acetylcysteine for 1 h and then incubated with 200 μM $ZnCl_2$ for 10 min. For $H_2O_2$ treatment, HNN8 cells were exposed to 500 μM $H_2O_2$ for 10 min. Cell lysates were assayed for PI3K activity as described under *Materials and Methods*. C, *N*-acetylcysteine suppresses $Zn^{2+}$-induced JNK stimulation. HNN8 cells were pretreated with 30 mM *N*-acetylcysteine for 1 h, and then exposed to 200 μM $ZnCl_2$ for 1 h. JNK1 activity in cell lysates was measured by immunocomplex kinase assay. D, *N*-acetylcysteine blocks $Zn^{2+}$-induced stimulation of the transcriptional activity of c-Jun. HNN8 cells were cotransfected with appropriate plasmids (pFR-Luc, pFA2-c-Jun, or pFC2 empty vector) along with pSV-β-gal. At 24 h after transfection, the cells were pretreated with 30 mM NAC for 1 h where indicated. Cells were then exposed to 100 μM $ZnCl_2$ for 2 h, incubated further for 8 h, and then assayed for luciferase activity as in Fig. 5.

molecule, whereas PI3K$\gamma$ misses the p85 subunit (Vanhaesebroeck and Waterfield, 1999). The $Zn^{2+}$-induced JNK stimulation was suppressed by a dominant negative mutant of PI3K$\gamma$ but not by a dominant negative mutant of PI3K$\alpha$ (data not shown). Thus, PI3K$\gamma$ seems to participate in the $Zn^{2+}$ action on the JNK pathway.

ROS plays a pivotal role in a variety of neuronal activities in the normal brain and in the pathogenesis of neurological disorders (Floyd, 1999). ROS generation has been detected in neurons under various conditions including excitotoxicity, nerve growth factor withdrawal, and hyperglycemia (Dugan et al., 1995; Greenlund et al., 1995; Li et al., 1999). $Zn^{2+}$ has been reported to induce the production of ROS in neuronal cells (Kim et al., 1999b). We confirmed the $Zn^{2+}$-induced ROS production in this study. Interestingly, ROS functions as an activating signal for the JNK pathway (Lo et al., 1996). Therefore, it is tempting to propose that $Zn^{2+}$ may induce stimulation of the JNK pathway through ROS generation. In fact, this proposition is consistent with our finding in this study that the free-radical scavengers NAC can block the $Zn^{2+}$-induced JNK stimulation.

JNK, when activated, can phosphorylate c-Jun, which is a component of the transcription factor complex AP-1 (Angel and Karin, 1991). The c-Jun phosphorylation by JNK results in the stimulation of the transcriptional activity of c-Jun (Ip and Davis, 1998). Therefore, the JNK stimulation by $Zn^{2+}$ may lead to an enhancement of the transcription stimulating activity of c-Jun. Indeed, our data using a luciferase reporter assay system show that exposure of cells to $Zn^{2+}$ resulted in an increase in the *trans*-activating activity of c-Jun. By inducing AP-1 stimulation, $Zn^{2+}$ could modulate the expression of a number of proteins in neural cells exposed to $Zn^{2+}$. Thus, understanding the activation of the JNK pathway and AP-1 by $Zn^{2+}$ will importantly contribute to our understanding of how $Zn^{2+}$ serves as a signaling mediator in normal brain function or in neurological diseases. In particular, translocation of $Zn^{2+}$ into postsynaptic neurons after seizure or hypoxic-ischemia may contribute to the activation of AP-1 and other $Zn^{2+}$-sensitive transcription factors in target neurons that plays a crucial role in the process of neuronal death or plasticity (Smeyne et al., 1993; Pennypacker et al., 1995; Domanska-Janik et al., 1999).

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