

# Selective Activation of the c-Jun N-Terminal Protein Kinase Pathway during 4-Hydroxynonenal-Induced Apoptosis of PC12 Cells

YUNJO SOH,<sup>1,2</sup> KYU-SHIK JEONG,<sup>1,3</sup> INSONG JAMES LEE,<sup>4</sup> MYUNG-AE BAE, YONG-CHUL KIM, and  
BYOUNG J. SONG

Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Rockville, Maryland (Y.S., K.-S.J., I.J.L., M.-A.B., B.J.S.); and Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland (Y.-C.K.)

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## ABSTRACT

The by-product of lipid peroxidation, 4-hydroxynonenal (HNE), was shown to cause apoptosis in PC12 cells. In this study, we investigated the molecular mechanism of HNE-induced apoptosis in these cells. Specifically, we determined the effect of HNE on the activities of mitogen-activated protein (MAP) kinases involved in early signal transduction. Within 15 to 30 min after HNE treatment, c-Jun N-terminal protein kinase (JNK) was maximally activated, before it returned to control level at 1 h post-treatment. In contrast, activities of extracellular signal-regulated kinase and p38 MAP kinase remained unchanged from their baseline levels. Stress-activated protein kinase kinase (SEK1), an upstream kinase of JNK, was also activated within 5 min after HNE treatment and remained activated for up to 60 min. Marked activation of the JNK pathway through SEK1 and apoptosis signal-regulating kinase 1 (ASK1), an upstream kinase of SEK1, was demonstrated by the transient transfection

of cDNA for wild-type SEK1 or ASK1 together with JNK into COS-7 cells. Furthermore, significant reductions in JNK activation and HNE-induced cell death were observed when either of the dominant negative mutant of SEK1 or ASK1 was cotransfected with JNK. Pretreatment of PC12 cells with a survival-promoting agent, 8-(4-chlorophenylthio)-cAMP, prevented both the HNE-induced JNK activation and apoptosis. Nonaldehyde, a nontoxic aldehyde, neither caused apoptosis nor JNK activation. Pretreatment of PC12 cells with SB203580, a specific inhibitor of p38 MAP kinase, had no effect on HNE-induced apoptosis. All these data suggest that the selective JNK activation by HNE is critical for the apoptosis of PC12 cells and that the HNE-mediated apoptosis is likely to be mediated through the activation of the ASK1-SEK1-JNK pathway without activation of extracellular signal-regulated kinase or p38 MAP kinase.

Reactive moieties produced during stressful conditions cause the oxidation of polyunsaturated fatty acids in membrane lipid bilayers. Without sufficient levels of defense mechanisms such as free radical scavengers or antioxidants, increasing levels of lipid hydroperoxides and peroxides can be produced by self-perpetuating chain reactions. Eventually, cytotoxic lipid aldehydes, including 4-hydroxynonenal (HNE), an end product of lipid peroxidation, is produced

(Esterbauer et al., 1991). These lipid peroxides can directly damage cells and tissues through interaction with cellular macromolecules, including proteins, lipids, and nucleic acids. For example, chronic alcohol consumption with high-iron diet (Tsukamoto et al., 1995) or high-fat diet (Nanji et al., 1994) and treatment with hepatotoxic agents markedly elevate the levels of lipid peroxides, including HNE and damage hepatocytes and the liver (Esterbauer et al., 1991; Kamimura et al., 1992). Despite the extensive detailing of the production of lipid peroxides under stressful conditions, the mechanisms by which lipid peroxides cause cell death have not been studied systematically.

Extracellular signal-regulated kinase (ERK) plays a major role in cell proliferation and differentiation as well as sur-

<sup>1</sup> Both authors contributed equally to the present work.

<sup>2</sup> Current address: Department of Neuroscience, Graduate School of East-West Medical Science, Kyung-Hee University, Seoul, Korea.

<sup>3</sup> Current address: Korea Research Institute of Bioscience and Biotechnology, Taejeon, Korea.

<sup>4</sup> Current address: EpiGenesis Pharmaceuticals, Inc., 2005 Eastpark Blvd., Cranbury, NJ.

**ABBREVIATIONS:** HNE, 4-hydroxy-2-nonenal; ERK, extracellular signal-regulated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal protein kinase; p38 MAP kinase, p38 mitogen-activated protein kinase; SEK1, SAPK kinase; MAP kinase, mitogen-activated protein kinase; MEKK, MAP kinase kinase kinase; ASK1, apoptosis signal-regulating kinase 1; HA, hemagglutinin; DAPI, 4,6-diamidino-2-phenylindole; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate; wt, wild type; ATF-2, activator of transcription factor-2; MTS, 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium; MKK, MAP kinase kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; AP-1, activator protein 1; GST, glutathione S-transferase.

vival mediated by various growth factors (Karin, 1995). However, the stress-activated protein kinase (SAPK) or c-Jun N-terminal protein kinase (JNK) (Derijard et al., 1994; Kyriakis et al., 1994) and p38 mitogen-activated protein kinase (p38 MAP kinase) (Han et al., 1994; Lee et al., 1994) are activated by various inflammatory cytokines and environmental stressors, often in a coordinated fashion (Raingeaud et al., 1995). JNK was recently shown to be an essential component of a signal transduction pathway that leads to apoptosis (Xia et al., 1995; Chen et al., 1996). In addition, JNK has been implicated in the induction of differentiation (Heasley et al., 1996) and oncogenesis (Raitano et al., 1995), depending on the microenvironment of the target cells. It is well established that JNK is activated through upstream protein kinases, including SAPK kinase (SEK1) (JNKK1 or MKK4) and mitogen-activated protein (MAP) kinase kinase 1 (MEKK1) or apoptosis signal-regulating kinase 1 (ASK1), whereas p38 MAP kinase is activated by MAP kinase kinase 3 (MKK3) (MKK6 or SEK2) and ASK1 (Derijard et al., 1994; Raingeaud et al., 1995; Ichijo et al., 1997).

Apoptosis or cell damage under various stressful conditions is generally thought to be mediated through rapid activation of a family of SAPK (JNK) and p38 MAP kinase with little change or reduction in the activity of ERK. Recent data demonstrated that HNE caused time-dependent apoptosis of PC12 cells and primary cells from the hippocampus (Kruman et al., 1997). These cells, however, were not sensitive to HNE structural analogs such as 2-nonenal, nonaldehyde, or other lipid peroxides, including malondialdehyde. Because the molecular mechanism of HNE-mediated cell death has not been investigated, this study was undertaken to elucidate the mechanism of HNE-induced cell death by measuring the activities of the MAP kinases involved in early signal transduction pathways. Our data indicate that cell death caused by HNE is mediated through selective activation of the JNK-SEK1-ASK1 pathway without alteration of the activity of ERK or p38 MAP kinase. This result is interesting because the p38 MAP kinase is often up-regulated by stressful conditions or by various cytotoxic compounds (Han et al., 1994; Lee et al., 1994; Raingeaud et al., 1995). In addition, this selective activation of JNK activity seems to be critically important in HNE-induced apoptosis of PC12 cells.

## Experimental Procedures

**Materials.** Specific monoclonal or polyclonal antibody to JNK1, ERK2, p38 MAP kinase, phospho-p38 MAP kinase or hemagglutinin (HA) was from PharMingen (San Diego, CA), New England Biolabs (Beverly, MA), Upstate Biotechnology, Inc. (Buffalo, NY), or Santa Cruz Biotechnology (Santa Cruz, CA), respectively. 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes, Inc. (Eugene, OR). Myelin basic protein, type I rat tail collagen, and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were purchased from Sigma (St. Louis, MO). The plasmids pcDNA3-HA-JNK1, pEBG-SEK1 wild type (wt), and its dominant negative mutant pEBG-SEK1 KR (Lys → Arg) were from Dr. J. S. Gutkind (National Institute on Dental Research, National Institutes of Health, Bethesda, MD); cDNA for glutathione *S*-transferase-activator of transcription factor-2 (GST-ATF2) fusion protein from Dr. E. J. Choi (Korea University, Seoul, Korea); and cDNA for pcDNA3-ASK1-HA wt and dominant negative mutant pcDNA3-ASK1 KR-HA from Dr. H. Ichijo (Japanese Foundation for Cancer Research, Tokyo, Japan). HNE was synthesized and purified over a silica gel column and its concentration was calculated from the optical density as described (Es-

terbauer and Weger, 1967). SB203580 was provided by Dr. J. Lee (SmithKline Beecham, King of Prussia, PA). Cell culture media were purchased from Life Technologies Inc. (Gaithersburg, MD). Other reagents not listed were the same as described in Soh et al. (1998).

**Cell Culture.** PC12 cells were grown on collagen-precoated plastic microtiter plates or culture dishes in RPMI 1640 containing 5% heat-inactivated fetal bovine serum, 10% horse serum, and antibiotics (100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 100 U of fungizone) at 37°C in 5% CO<sub>2</sub> and 95% air in a humidified incubator. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

**Measurement of HNE-Induced Cytotoxicity.** PC12 cells ( $2 \times 10^4$  cells/well) were grown in collagen-precoated 96-well microtiter plates for 2 days in regular serum-containing medium. After cells were exposed to low-serum-containing medium (1% horse serum and 1% fetal bovine serum) for 16 h, varying concentrations of HNE (diluted in ethanol, 0.05% as a final concentration) were added to the culture medium. After incubation of PC12 cells for indicated times, HNE-containing medium was aspirated from the plates and each well was washed twice with  $1 \times$  PBS. Viability of remaining PC12 cells was determined as described in the protocol supplied with the CellTiter 96 NonRadioactive Cell Proliferation Assay kit (Promega, Madison, WI) with 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium (MTS) as a substrate. The optical density at 495 nm was determined with an automatic enzyme-linked immunosorbent assay reader. In some cases, cell viability also was determined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983).

**Immunocomplex Kinase Activity Assay.** PC12 or COS-7 cells treated with HNE for indicated times were harvested and homogenized in ice-cold lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 12.5 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1  $\mu$ g/ml leupeptin. Cell debris and particulate fractions were removed by centrifugation at 14,000g for 10 min at 4°C. The catalytic activity of JNK1, ERK, or p38 MAP kinase in the remaining soluble fraction (200  $\mu$ g/reaction) was performed after immunoprecipitation with the respective antibody (a mouse monoclonal antibody to JNK1, a polyclonal antibody to ERK2, or a polyclonal antibody to p38 MAP kinase) according to a published method (Chen et al., 1996). The kinase reaction buffer contained 20 mM HEPES (pH 7.4), 1 mM  $\beta$ -glycerophosphate, 7.5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 0.05 mg/ml substrate protein, 20  $\mu$ M ATP, and 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP. Purified GST-ATF2 fusion protein or ATF-2 protein (Santa Cruz Biotechnology) was used as a protein substrate for JNK1 and MBP for both ERK and p38 MAP kinase. The reaction was initiated by the addition of radiolabeled ATP to the reaction mixture. After incubation for 30 min at 30°C, the enzyme reaction was stopped by  $1 \times$  SDS sample buffer and the reaction mixtures were subjected to electrophoresis on 12% SDS-polyacrylamide gels followed by staining with Coomassie blue, drying, and autoradiography with intensifying screens. In some cases, proteins from cell extracts were subjected to immunoblot analysis with the antibody against the respective MAP kinase to verify their relative expression by enhanced chemiluminescence reaction with a SuperSignal West Pico ECL detection kit (Pierce Chemicals, Rockford, IL).

**Transient Expression of Transfected cDNAs.** The respective cDNA constructs for HA-JNK, SEK1 wt, HA-ASK1 wt, dominant negative mutant, SEK1 KR, or HA-ASK1 KR were cotransfected into COS-7 cells (60–70% confluence) with LipofectAMINE reagent according to the manufacturer's instruction (Life Technologies). In each dish (90-mm diameter), 2.5  $\mu$ g of cDNA of pcDNA3-HA-JNK and 2.5  $\mu$ g of cDNA of pEBG-SEK1 wt or pEBG-SEK1 KR mutant were cotransfected with 1.0  $\mu$ g of cDNA of HA-ASK1 wt or dominant HA-ASK1 KR mutant as indicated in the figure legend. Efficiency of DNA transfection was determined in two independent experiments by calculating the percentage of immunostained cells over the total

number of transfected COS-7 cells after fixation of cells with 4% paraformaldehyde (pH 7.4) for 30 min. Immunostaining of fixed cells was performed by stepwise incubation with a primary antibody against the respective protein of HA, JNK, SEK1, or ASK1 protein (Santa Cruz Biotechnology) followed by avidin and biotinylated horseradish peroxidase-conjugated secondary antibody (Vectastain ABC kit and VIP substrate; Vector Laboratories, Burlingame, CA).

**Treatment of PC12 Cells with CPT-cAMP, Nonaldehyde, or SB203580.** PC12 cells grown to 60 to 70% confluence in collagen-precoated culture dishes (90-mm diameter) or 96-well microtiter plates were treated with 0.5 mM CPT-cAMP, 25  $\mu$ M nonaldehyde, or SB203580 (10 or 50  $\mu$ M) for 30 min before and during treatment with 25  $\mu$ M HNE for another 15 or 30 min (for JNK activity assay) and 16 to 24 h (for cell viability assay). PC12 cells were subjected to either JNK activity assay after cell harvest or cell viability measurement as described above.

**Statistical Analysis.** All the experimental data shown were repeated at least three times, unless otherwise indicated. The data were analyzed by Mann-Whitney *U* test and *P* < .05 was considered significant.

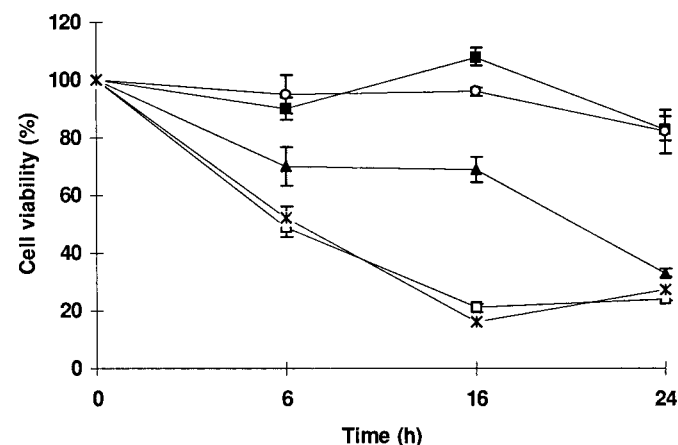
## Results

**Cytotoxic Effect of HNE on PC12 Cell Death.** To determine the cytotoxic effects of HNE on PC12 cells, we treated PC12 cells with varying HNE concentration for different incubation times and measured cell death with the MTS cell viability assay. A significant portion of cells died after HNE treatment for up to 24-h incubation in dose- and time-dependent manners. As shown in Fig. 1, approximately 30.3, 30.0, and 67.8% of PC12 cells died on treatment with 25  $\mu$ M HNE for 6, 16, and 24 h of incubation, respectively, whereas less than 10% of PC12 cells died at 5 and 10  $\mu$ M HNE under our experimental conditions. The rates of cell death were significantly increased at higher concentrations of HNE. Approximately 75 to 85% of PC12 cells died after treatment with 50 and 100  $\mu$ M HNE for 16 and 24 h. Similar levels of HNE-induced cell death were observed by using the MTT cell death assay (data not shown).

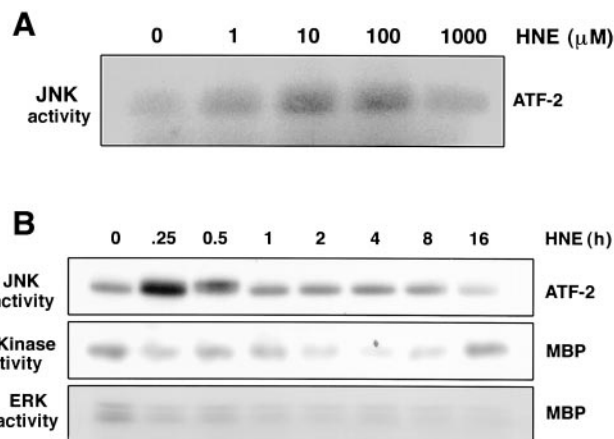
**HNE-Dependent Apoptosis.** To verify the type of cell death, we stained the cells with DAPI, a sensitive assay for apoptosis. Without HNE treatment, the nuclei of control cells showed uniform staining, indicating that cells were healthy

and nuclei intact. In contrast, after 24-h treatment with 25  $\mu$ M HNE, more than a half of PC12 cells exhibited typical characteristics of apoptosis, such as nuclear condensation as determined by DAPI staining (data not shown), consistent with the previous observation by Kruman et al. (1997). These data confirm that HNE-induced cell death is mediated via apoptosis.

**Dose- and Time-Dependent Selective Activation of JNK by HNE.** To investigate the mechanism of HNE-induced apoptosis of PC12 cells, we studied the time- and dose-dependent effects of HNE on the catalytic activities of MAP kinases involved in early signal transduction: JNK, p38 MAP kinase, and ERK. Throughout our experiments shown in this study, comparable amounts of immunoprecipitated kinase proteins and their substrate proteins were always ensured by Coomassie blue staining of SDS-polyacrylamide gels and immunoblot analyses with antibodies against the respective protein (data not shown but provided to the reviewers). As shown in Fig. 2A, JNK activity was slightly activated by 1  $\mu$ M HNE, whereas it was maximally (6.8-fold) activated by 10 and 100  $\mu$ M HNE. However, at 1 mM HNE, JNK activation was markedly reduced to 2.8-fold, possibly due to rapid necrosis of PC12 cells at this concentration of HNE. We chose to use 25  $\mu$ M HNE for all subsequent experiments because this HNE concentration resulted in maximal activation of JNK and is pathophysiologically relevant (Esterbauer et al., 1991). HNE treatment activated JNK 4.8-fold within 15 to 30 min. The level of JNK activity returned to the background level by 60 min (Fig. 2B). However, the low basal activities of ERK and p38 MAP kinase remained unchanged throughout the HNE treatment period. This unexpected result was verified with another substrate, ATF-2, for p38 MAP kinase in the assay (Raingeaud et al., 1995). These data suggest that HNE is likely to mediate its cytotoxic effect in PC12 cells through a selective activation of the JNK pathway. Under our experimental conditions, JNK itself did not appear to be the direct target of HNE because HNE did not increase the activity of JNK immobilized on protein G agarose beads (data not shown) or when produced in COS-7 cells (Fig. 3B).

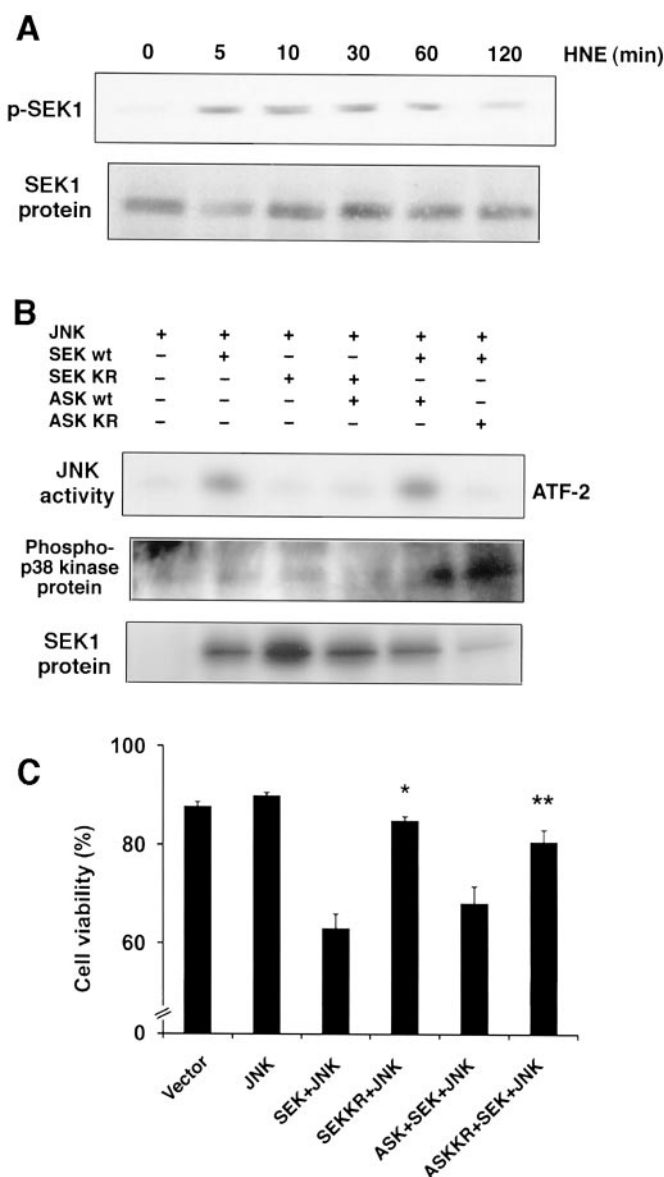


**Fig. 1.** Time- and HNE concentration-dependent apoptosis of PC12 cells. Time- and HNE concentration-dependent cell viability were determined by the method described under *Experimental Procedures* with MTS: ■, 5  $\mu$ M HNE; ○, 10  $\mu$ M HNE; ▲, 25  $\mu$ M HNE; □, 50  $\mu$ M HNE; and \*, 100  $\mu$ M HNE. Each point represents the average of five determinants with standard errors.



**Fig. 2.** Time- and HNE concentration-dependent selective activation of JNK. A, JNK activity was measured 15 min after treatment with varying concentrations of HNE as indicated. B, catalytic activities of JNK, ERK, and p38 MAP kinase in the PC12 cell extracts were determined by the immunocomplex kinase assay method, following treatment with 25  $\mu$ M HNE for indicated times. Each radiolabeled protein was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.





**Fig. 3.** Activation of SEK1 and ASK1 by HNE. **A**, time-dependent phosphorylation of SEK1 by HNE in PC12 cells. PC12 cells were treated with 25  $\mu$ M HNE for indicated times, harvested, and homogenized as described under *Experimental Procedures*. Equal amounts of soluble fraction (200  $\mu$ g/well) from each sample were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. The membrane was then exposed to antibody against phospho-SEK1 (top) or SEK1 (bottom). The antigen recognized by the primary antibody was visualized by enhanced chemiluminescence method. **B**, activation of the JNK-SEK1-ASK1 pathway by HNE in transfected COS-7 cells. Expression vectors, containing the cDNAs for HA-JNK, SEK1, and ASK1 wt or dominant mutant in different combinations as indicated, were transiently cotransfected into COS-7 cells grown in 96-mm culture dishes. Cells were then treated with 25  $\mu$ M HNE for 30 min, harvested, homogenized, and immunoprecipitated with HA antibody before the measurement of JNK activity (top). Equal amounts of protein (300  $\mu$ g/well) were used to determine the level of phospho-p38 MAP kinase (middle) or SEK1 protein (bottom) by immunoblot analysis. **C**, role of activation of the JNK pathway in HNE-induced apoptosis of transfected COS-7 cells. COS-7 cells ( $2 \times 10^5$  cells/well) grown in 96-well microtiter plates were transiently transfected with 0.125  $\mu$ g of expression constructs as indicated in **B**, allowed to grow overnight in low-serum-containing medium, and treated with 25  $\mu$ M HNE for 24 h. Cell viability was determined by MTT method as described under *Experimental Procedures*. \* and \*\*, significantly different ( $P < .02$ ) from the corresponding SEK1 wt and ASK1 wt, respectively.

**Phosphorylation of SEK1 by HNE.** To characterize the pathway of JNK activation by HNE, the effect of HNE on the phosphorylation (activation) status of the immediate upstream kinase of JNK, SEK1 (also called JNKK1 or MKK4), was analyzed. A polyclonal antibody specific to phospho-SEK1 or SEK1 protein (New England Biolabs) was used for the assay. As shown in Fig. 3A (top), the level of phosphorylated SEK1 protein was rapidly increased within 5 min after treatment of PC12 cells with 25  $\mu$ M HNE and the elevated SEK1 level persisted for approximately 60 min after HNE treatment. However, the levels of SEK1 protein during the HNE treatment were unchanged, as verified by immunoblot result with an antibody against SEK1 protein (Fig. 3A, bottom).

**Activation of JNK1-SEK1-ASK1 pathway by HNE.** We then tested whether the activation of JNK by HNE was mediated through the activation of SEK1 and ASK1, an immediate upstream activator of both SEK1 and MKK3 or MKK6 (p38 MAP kinase activators). COS-7 cells were used for the following experiments because of their higher transfection rates than that for PC12 cells (data not shown). COS-7 cells were transiently transfected with various combinations of expression constructs for HA-JNK, SEK1 wt, ASK1 wt as well as their corresponding dominant mutants (SEK1 KR and ASK1 KR). Although the transfection efficiency of each cDNA ranged from 13 to 28% of total cells, cotransfection of multiple DNAs did not change the transfection efficiency and we were able to observe the effects of transfected DNAs. When the cDNA for HA-JNK was transfected alone into COS-7 cells, very low basal JNK activity was observed and HNE treatment did not increase the activity of JNK, indicating that HNE does not activate JNK directly (Fig. 3B, top, lane 1). When SEK1 wt was cotransfected with JNK, the activation of JNK by HNE was observed (lane 2), indicating that SEK1 activates JNK on HNE exposure. Cotransfection of SEK1 KR mutant with JNK efficiently blocked the HNE mediated JNK activation (lane 3). The presence of ASK1 wt could not overcome the SEK1 KR mutant because no activation of JNK was observed when ASK1 wt was cotransfected with SEK1 KR and JNK (lane 4). Cotransfection of the dominant mutant ASK1 KR with SEK1 wt and JNK markedly diminished the HNE-induced JNK activation (lane 6), indicating the sequential activation of ASK1, SEK1, and then JNK by HNE. Cotransfection of ASK1 wt along with SEK1 wt and JNK resulted in JNK activation on HNE treatment (lane 5). In contrast, HNE treatment did not phosphorylate (activate) p38 MAP kinase (Fig. 3B, middle), despite the presence of JNK (data not shown) or SEK1 protein (Fig. 3B, bottom) expressed in the transfected COS-7 cells, as confirmed by immunoblot and immunostaining of the fixed cells (data not shown but provided to the reviewers) with the respective antibody against HA, JNK, or SEK1.

Similarly, expression of ASK1 protein was only observed in the COS-7 cells transfected with ASK1 wt or mutant cDNA, as verified by immunostaining with antibody against ASK1 (data not shown but provided to the reviewers). These data establish that HNE increases JNK activity through the ASK1-SEK1-JNK pathway and that HNE does not directly activate JNK.

**Critical Role of JNK Activation in HNE-Induced Cell Death.** To determine the role of JNK activation in HNE-induced cell death, we transiently transfected the same DNA

constructs into COS-7 cells grown in 96-well microtiter plates. COS-7 cells were then exposed to 25  $\mu$ M HNE for 24 h before cell viability was measured by MTT assay. As shown in Fig. 3C, approximately 12.2 and 10.0% of cells died on exposure to HNE with pcDNA3 vector alone and HA-JNK plasmid, respectively. In contrast, 36.6% of cells died when SEK1 wt was cotransfected with HA-JNK. A significant reduction in cell death was observed with the dominant mutant, SEK1 KR (14.3%), compared with that with SEK1 wt. A similar pattern of results was observed with ASK1 wt (32.1% cell death) and its corresponding ASK1 KR mutant (19.1% cell death). In contrast, a small portion (average of 6.6%) of transfected COS-7 cells died in the absence of HNE treatment. These results clearly establish an important role of JNK activation in HNE-induced cell death.

**Effect of CPT-cAMP or Nonaldehyde on HNE-Induced JNK Activation and Cell Death.** Recent studies suggested that a neurosurvival agent, CPT-cAMP, can promote survival of PC12 cells (Park et al., 1996; Hartfield et al., 1998) as well as other neuronal cells (Virdee et al., 1997) from apoptosis caused by withdrawal of neurotrophic factors. This agent was shown to promote cell survivals by acting upstream of JNK to prevent JNK activation (Park et al., 1996). We tested the potential for CPT-cAMP to protect PC12 cells from HNE-induced apoptosis as well as to inhibit the HNE-induced JNK activation. Consistent with our previous results (Fig. 2B), HNE treatment for 15 and 30 min caused rapid activation of JNK by HNE (Fig. 4A, lanes 2 and 3), compared with the solvent control (lane 1). CPT-cAMP alone did not appear to affect the JNK activity (lane 4). Pretreatment of

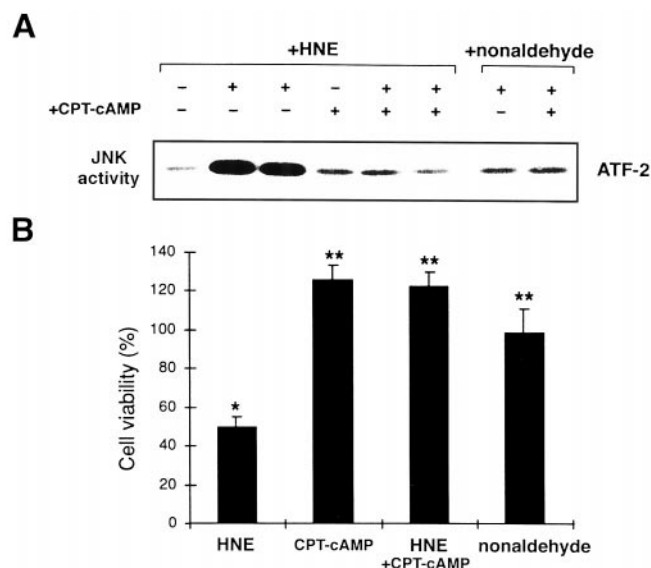
PC12 cells with CPT-cAMP for 30 min before HNE treatment markedly reduced the HNE-induced JNK activation at 15 and 30 min after HNE treatment (Fig. 4A, lanes 5 and 6). In contrast, treatment of 25  $\mu$ M nonaldehyde, which did not cause apoptosis of PC12 cells (Kruman et al., 1997), did not affect the activity of JNK (lanes 7 and 8).

Pretreatment of PC12 cells with CPT-cAMP completely prevented the HNE-induced apoptosis of PC12 cells (Fig. 4B). In addition, 25  $\mu$ M nonaldehyde, which did not activate JNK (Fig. 4A), did not cause cell death of PC12 cells (Fig. 4B). Taken together, these data indicate that an early JNK activation by HNE appears to be necessary for HNE-induced apoptosis of PC12 cells.

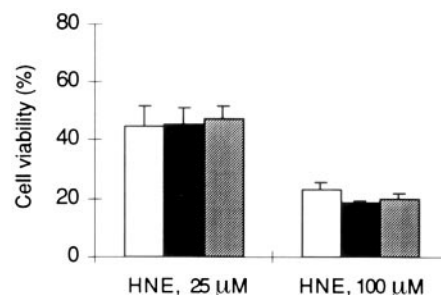
**Effect of SB203580 on HNE-induced Cell Death.** Horstmann et al. (1998) recently reported that specific inhibitors of p38 MAP kinase, SB203580 and SB202190, could promote survival of PC12 cells against apoptosis caused by withdrawal of growth factors. To test the possible role of p38 MAP kinase in HNE-mediated apoptosis, we pretreated PC12 cells with SB203580 before HNE treatment. At two different concentrations of HNE, pretreatment with either 10 or 50  $\mu$ M SB203580 did not change the percentage of cells undergoing apoptosis (Fig. 5). These data together with our earlier result (Fig. 2B) indicate that p38 MAP kinase is not involved in HNE-mediated apoptosis of PC12 cells.

## Discussion

Under pathological or other stressful conditions where reactive species are produced, cellular macromolecules such as DNA, proteins, and lipids are modified into DNA adducts, oxidized proteins, and lipid peroxides, respectively. From the lipid peroxidation of plasma membrane, reactive lipid aldehydes such as HNE or hydroxyhexanal are produced via enzymatic and nonenzymatic reactions (Esterbauer et al., 1991). Newly synthesized HNE can readily move across cell membranes and can interrupt the normal functions of proteins as well as DNAs (Esterbauer et al., 1991; Martelli et al., 1994). Under extreme conditions of stress, concentration of newly synthesized HNE may reach up to 100  $\mu$ M (Esterbauer et al., 1991) and thus, HNE may serve as a common cytotoxic signal in many cell types and tissues. For example, the levels of HNE-bound proteins are increased selectively in neurons in the substantia nigra of Parkinson's patients (Yoritaka et al., 1996) and levels of free HNE are increased more than



**Fig. 4.** Effects of CPT-cAMP and nonaldehyde on HNE-induced JNK activation and apoptosis. A, PC12 cells were pretreated with 0.5 mM CPT-cAMP for 30 min before treatment with 25  $\mu$ M HNE or nonaldehyde for 15 and 30 min. Immediately after HNE treatment, cells were harvested and the activities of JNK in different cell extracts were determined as described in Fig. 2 legend. B, another set of PC12 cells ( $2 \times 10^4$  cells/well) were grown in 96-well microtiter plates for 2 days followed by exposure to a low-serum-containing medium for 16 h. After treatment with HNE for 16 h in the absence and presence of 0.5 mM CPT-cAMP, as indicated, cell viability was determined and expressed as percentage of control (without HNE treatment) in bar graphs. Nonaldehyde was treated for 16 h in the absence of CPT-cAMP, before determination of cell viability. \* and \*\*, significantly different ( $P < .001$ ) from the vehicle control and the HNE-treated sample, respectively.



**Fig. 5.** Effect of SB203580 on HNE-induced apoptosis. PC12 cells were grown in microtiter plates for 2 days and treated with a low-serum-containing medium for 16 h. Cells were then exposed to vehicle alone ( $\square$ ), 10  $\mu$ M ( $\blacksquare$ ), or 50  $\mu$ M ( $\boxplus$ ) of SB203580 for 30 min before and during treatment with 25 or 100  $\mu$ M HNE for additional 16 h, as indicated. Cell viability was then determined by the MTS viability assay.

2-fold in the cerebrospinal fluid of Alzheimer's disease patients (Markesbery and Lovell, 1998).

Despite numerous reports describing HNE protein adducts, the mechanism by which HNE interferes with cellular functions is not well known. To study the molecular process of HNE-induced cell death, we investigated HNE-mediated changes in the activities of MAP kinases involved in cell survival and death. Parola et al. (1998) demonstrated that HNE directly increased the JNK activity in human hepatic stellate cells. Our data indicate that HNE selectively activated JNK activity by activating its upstream kinases, SEK1 and ASK1. Because ASK1 can phosphorylate both SEK1 (JNKK1 or MKK4) and MKK3 (MKK6), an upstream protein kinase of p38 MAP kinase (Raingeaud et al., 1995; Ichijo et al., 1997), it may be expected that p38 MAP kinase would be activated by HNE. However, no significant change in the activity of p38 MAP kinase or ERK was observed, despite testing under extensive experimental conditions. Our results of the absence of p38 MAP kinase activation by HNE in PC12 cells and transfected COS-7 cells is in contrast with the data by Uchida et al. (1999) who recently reported that HNE not only activated JNK but also increased p38 MAP kinase activity in rat liver epithelial RL34 cells. It has been reported that p38 MAP kinase and JNK are often up-regulated in a coordinated fashion by various cytotoxic agents such as cytokines, including tumor necrosis factor- $\alpha$ , interleukin-1, UV and x-rays, toxic chemicals, and H<sub>2</sub>O<sub>2</sub> (Raingeaud et al., 1995; Chen et al., 1996). The lack of activation of p38 MAP kinase by HNE in the current study was not due to the absence of this enzyme in PC12 cells because it was shown that arsenite can stimulate its activity in parallel with the JNK activity in these cells (Liu et al., 1996) and our immunoblot data (data not shown but provided to the reviewers). Furthermore, both SEK1 and MKK3 (MKK6 or SEK2) in PC12 cells were shown to be stimulated by osmotic shock, UV-irradiation, and an inhibitor of protein synthesis, anisomycin (Meier et al., 1996), suggesting the presence of a functionally intact p38 MAP kinase-SEK2 pathway in these cells. Thus, our results, taken together with previous results (Raingeaud et al., 1995; Parola et al., 1998; Uchida et al., 1999) suggest that the activation of p38 MAP kinase is dependent on cell type as well as the stressors used. The lack of p38 MAP kinase activation by HNE in PC12 cells could result from higher levels or more active PAC1 (Raingeaud et al., 1995) or MKP-1, which specifically dephosphorylates phospho-p38 MAP kinase (Mendelson et al., 1996). This possibility is currently under investigation in our laboratory. The selective activation of the JNK pathway by HNE is similar to that observed after treatment with hepatotoxic agents such as CCl<sub>4</sub> (Mendelson et al., 1996) and acetaminophen (our unpublished data).

The question of whether JNK activation or c-Jun phosphorylation is absolutely necessary for the cell death has been actively debated. In some cells such as those from *c-fos* or *c-Jun* null embryos (Roffler-Tarlov et al., 1996) and sympathetic motor neurons (Virdee et al., 1997), JNK activation or phosphorylated c-Jun may not be a causal factor in apoptosis. However, in other types of cell such as PC12 cells, JNK activation seems to be sufficient for apoptosis because activation of activator protein-1 (AP-1) activity and overexpression of c-Jun wt, SEK1 wt, or ASK1 wt protein led to cell death (Ham et al., 1995; Ichijo et al., 1997; Luo et al., 1998)

or counteracted the antiapoptotic effect of bcl-2 (Park et al., 1997). Furthermore, overexpression of dominant negative mutant of c-Jun, SEK1 KR, and ASK1 KR prevented apoptotic cell death caused by withdrawal of nerve growth factor (Ham et al., 1995), dopamine (Luo et al., 1998), and tumor necrosis factor- $\alpha$  (Ichijo et al., 1997). Additionally, JNK activation appears to be an early event for apoptosis of PC12 cells by ceramide (Hartfield et al., 1998), manganese (Hirata et al., 1998), and withdrawal of survival factors (Le-Niculescu et al., 1999). It is also true that motor neuron apoptosis is blocked by a novel inhibitor of the JNK-signaling pathway (Maroney et al., 1998). Therefore, whether JNK activation is a prerequisite for cell death seems to depend on the cell type, cell death signal, the duration of JNK activation, and the cellular environment, as proposed (Chen et al., 1996; Hazzalin et al., 1996; Virdee et al., 1997). Within our experimental system, the data clearly support the proapoptotic role of JNK pathway and demonstrate that activation of the JNK pathway is necessary for HNE-induced cell death.

During JNK activation, c-Jun protein is phosphorylated and heterodimerizes with another protein, c-fos, to produce the transcription factor AP-1, which interacts with specific DNA sites and thus regulates the transcription of various genes. Camandola et al. (2000) recently reported that HNE caused apoptosis of embryonic neuronal cells through the activation of JNK, AP-1, and caspases, suggesting an important role of JNK activation in neuronal injury. Our preliminary result from electrophoretic mobility shift assay revealed a similar activation of AP-1-binding activity at 1 and 8 h after HNE treatment in PC12 cells. Thus, our data support that AP-1 is also activated in PC12 cells by HNE through activation of the JNK pathway.

Numerous reports have demonstrated that HNE inhibits functions of various cellular proteins (for review, see Esterbauer et al., 1991). Our data clearly demonstrate that HNE can also stimulate the ASK1-SEK1-JNK pathway rapidly and selectively. Because HNE did not appear to directly activate JNK on agarose bead or HA-JNK expressed alone in COS-7 cells (our current data), HNE may act directly on the immediate upstream kinase of SEK1, either ASK1 or MEKK1. Although we do not know the mechanism of direct activation of either ASK1 or MEKK1 by HNE, its effect could be mediated through the various small GTP-binding proteins such as Rho or Rac. Alternatively, HNE-mediated activation of ASK1 or SEK1 could result from the inhibition of a specific phosphatase, which selectively dephosphorylates the phospho-ASK1 or phospho-SEK1. These possibilities are being explored in this laboratory.

In conclusion, the data presented in this study demonstrate the sequential activation of the ASK1-SEK1-JNK pathway by HNE. Our data suggest that JNK activation without concurrent stimulation of p38 MAP kinase is necessary for the HNE-mediated apoptosis of PC12 cells.

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## References

- Camandola S, Poli G and Mattson MP (2000) The lipid peroxidation product 4-hydroxy-2,3-nonenal increase AP-1 binding activity through caspase activation in neurons. *J Neurochem* **74**:159–168.
- Chen Y-R, Wang X, Templeton D, Davis RJ and Tan T-H (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* **271**:31929–31936.
- Derijard B, Hibi M, Wu I-H, Barrett T, Su B, Deng T, Karin M and Davis RJ (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**:1025–1037.
- Esterbauer H, Schaur RJ and Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radic Biol Med* **11**:81–128.
- Esterbauer H and Weger W (1967) Über die Wirkungen von aldehyden auf gesunde und maligne zellen, 3. Mitt: Synthese von homologen 4-hydroxy-2-alkenalen. *Monatsh Chem* **98**:1994–2000.
- Ham J, Babji C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M and Rubin LL (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14**:927–939.
- Han J, Lee J-D, Bibbs L and Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science (Wash DC)* **265**:808–811.
- Hartfield PJ, Bilney AJ and Murray AW (1998) Neurotrophic factors prevent ceramide-induced apoptosis downstream of c-Jun N-terminal kinase activation in PC12 cells. *J Neurochem* **71**:161–169.
- Hazzalin CA, Cano E, Cuenda A, Barratt MJ, Cohen P and Mahadevan LC (1996) p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. *Curr Biol* **6**:1028–1031.
- Heasley LE, Storey B, Fanger GR, Butterfield L, Zamarripa J, Blumberg D and Maue RA (1996) GTPase-deficient G alpha 16 and G alpha q induce PC12 cell differentiation and persistent activation of c-Jun NH2-terminal kinases. *Mol Cell Biol* **16**:648–656.
- Hirata Y, Adachi K and Kiuchi K (1998) Activation of JNK pathway and induction of apoptosis by manganese in PC12 cells. *J Neurochem* **71**:1607–1615.
- Horstmann S, Kahle PJ and Borasio GD (1998) Inhibitors of p38 mitogen-activated protein kinase promote neuronal survival in vitro. *J Neurosci Res* **52**:483–490.
- Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science (Wash DC)* **275**:90–94.
- Kamimura S, Gaal K, Britton RS, Bacon BR, Triadafilopoulos G and Tsukamoto H (1992) Increased 4-hydroxynonenal levels in experimental alcoholic liver disease: Association of lipid peroxidation with liver fibrogenesis. *Hepatology* **16**:1014–1021.
- Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270**:16483–16486.
- Kruman I, Bruce-Keller AJ, Bredesen D, Waeg G and Mattson MP (1997) Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J Neurosci* **17**:5089–5100.
- Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Ruble EA, Ahmad MF, Avruch J and Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature (Lond)* **369**:156–160.
- Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher SM, Livi GP, White JR, Adams JI and Young PR (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (Lond)* **372**:739–746.
- Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR and Karin M (1999) Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol Cell Biol* **19**:751–763.
- Liu Y, Guyton KZ, Gorospe M, Xu Q, Lee JC and Holbrook NJ (1996) Differential activation of ERK, JNK/SAPK and P38/CSBP/RK map kinase family members during the cellular response to arsenite. *Free Radic Biol Med* **21**:771–781.
- Luo Y, Umegaki H, Wang X, Abe R and Roth GS (1998) Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. *J Biol Chem* **273**:3756–3764.
- Marksberry WR and Lovell MA (1998) Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiol Aging* **19**:33–36.
- Maroney AC, Glicksman MA, Basma AN, Walton KM, Knight E Jr, Murphy CA, Bartlett BA, Finn JP, Angeles T, Matsuda Y, Neff NT and Dionne CA (1998) Motor neuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. *J Neurosci* **18**:104–111.
- Martelli A, Canonero R, Cavanna M, Ceradelli M and Marinari UM (1994) Cytotoxic and genotoxic effects of five n-alkanals in primary cultures of rat and human hepatocytes. *Mutat Res* **323**:121–126.
- Meier R, Rouse J, Cuenda A, Nebreda AR and Cohen P (1996) Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase homologues in PC12 and KB cells. *Eur J Biochem* **236**:796–805.
- Mendelson KG, Contois L-R, Tevosian SG, Davis RJ and Paulson KE (1996) Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. *Proc Natl Acad Sci USA* **93**:12908–12913.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**:55–63.
- Nanji AA, Zhao S, Sadrzadeh SMH, Dannenberg AJ, Tahan SR and Waxman DJ (1994) Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats. *Alcohol Clin Exp Res* **18**:1280–1285.
- Park DS, Stefanis L, Yan CYI, Farinelli SE and Greene LA (1996) Ordering the cell death pathway. Differential effects of BCL2, an interleukin-1-converting enzyme family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth factor-deprived PC12 cells. *J Biol Chem* **271**:21898–21905.
- Park J, Kim I, Oh YJ, Lee K, Han PL and Choi EJ (1997) Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J Biol Chem* **272**:16725–16728.
- Parola M, Robino G, Marra F, Pinzani M, Bellomo G, Leonarduzzi G, Chiarugi P, Camandola S, Poli G, Waeg G, Gentilini P and Dianzani MU (1998) HNE interacts directly with JNK isoforms in human hepatic stellate cells. *J Clin Invest* **102**:1942–1950.
- Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ and Davis RJ (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* **270**:7420–7426.
- Raitano AB, Halpern JR, Hambuch TM and Sawyers CL (1995) The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proc Natl Acad Sci USA* **92**:11746–11750.
- Roffler-Tarlov S, Brown JJ, Tarlov E, Stolarov J, Chapman DL, Alexiou M and Papaioannou VE (1996) Programmed cell death in the absence of c-Fos and c-Jun. *Development* **122**:1–9.
- Soh Y, Song BJ, Jeng J and Kallarakal AK (1998) Critical role of arg433 in rat transketolase activity as probed by site-directed mutagenesis. *Biochem J* **333**:367–372.
- Tsukamoto H, Horne W, Kamimura S, Niemel O, Parkkila S, Yi-Herttuala S and Brittenham GM (1995) Experimental liver cirrhosis induced by alcohol and iron. *J Clin Invest* **96**:620–630.
- Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y and Osawa T (1999) Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *J Biol Chem* **274**:2234–2242.
- Virdee K, Bannister AJ, Hunt SP and Tolkovsky AM (1997) Comparison between the timing of JNK activation, c-Jun phosphorylation, and onset of death commitment in sympathetic neurones. *J Neurochem* **69**:550–561.
- Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science (Wash DC)* **270**:1326–1331.
- Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER and Mizuno Y (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci USA* **93**:2696–2701.

**Send reprint requests to:** Byoung J. Song, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, 12420 Parklawn Dr., Rm. 425, Rockville, MD 20852. E-mail: bs7t@nih.gov