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

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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 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-
vival mediated by various growth factors (Karin, 1995). How-
however, the stress-activated protein kinase (SAPK) or c-Jun
N-terminal protein kinase (JNK) (Derijard et al., 1994; Kyri-
aki 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 environ-
mental stressors, often in a coordinated fashion (Rainegaud
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 differen-
tiation (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) (JNK1 or
MKK4) and mitogen-activated protein (MAP) kinase kinase
kinase 1 (MEKK1) or apoptosis signal-regulating kinase 1
(ASK1), whereas p38 MAP kinase is activated by MAP kina-
ses 3 (MKK3) (MKK6 or SEK2) and ASK1 (Derijard
et al., 1994; Rainegaud et al., 1995; Ichijo et al., 1997).
Apoptosis or cell damage under various stressful condi-
tions is generally thought to be mediated through rapid ac-
tivation of a family of SAPK (JNK) and p38 MAP kinase with
little change or reduction in the activity of ERK. Recent data
have demonstrated that HNE caused time-dependent apoptosis
of PC12 cells and primary cells from the hippocampus (Krum-
ament 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 mo-
lecular mechanism of HNE-mediated cell death has not been
investigated, this study was undertaken to elucidate the mecha-
nism of HNE-induced cell death by measuring the activi-
ties of the MAP kinases involved in early signal trans-
duction 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 con-
ditions or by various cytotoxic compounds (Han et al., 1994;
Lee et al., 1994; Rainegaud 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 fac-
tor-2 (GST-ATF2) fusion protein from Dr. E. J. Choi (Korea Univer-
sity, Seoul, Korea); and cDNA for pcDNA3-ASK1-HA wt and domi-
nant 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 con-
centration 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 in collagen-precoated plas-
tic microtiter plates or culture dishes in RPMI 1640 containing 5%
heat-inactivated fetal bovine serum, 10% horse serum, and antibiot-
icus (100 U/ml penicillin G, 100 µg/ml streptomycin, and 100 U of
fungizocin) at 37°C in 5% CO2 and 95% air in a humidified incubator.
COS-7 cells were grown in Dulbecco’s modified Eagle’s medium con-
taining 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

Measurement of HNE-Induced Cytotoxicity. PC12 cells (2 x
104 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 x 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-carboxymethoxy-
thiophenyl)-2-(4-sulphophenyl)-2-tetrazolium (MTS) as a substrate.
The optical density at 495 nm was determined with an automatic
enzyme-linked immunosorben 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
were treated with HNE for indicated times were harvested and homo-
geinized 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 β-glycerophosphate,
1 mM Na3VO4, and 1 µg/ml leupeptin. Cell debris and particulate
fractions were removed by centrifugation at 14,000 g for 10 min at
4°C. The catalytic activity of JNK1, ERK, or p38 MAP kinase in the
remaining soluble fraction (200 µg/reaction) was performed after
immunoprecipitation with the respective antibody (a mouse mono-
clonal antibody to JNK1, a polyclonal antibody to ERK2, or a poly-
clonal 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 β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM
EGTA, 0.5 mM NaF, 0.5 mM Na3VO4, 0.05 mg/ml substrate protein,
20 µM ATP, and 2 µCi [γ-32P]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 radio
labeled ATP to the reaction mixture. After incubation for 30 min at 30°C,
the enzyme reaction was stopped by 1 x SDS sample buffer and the
reaction mixtures were subjected to electrophoresis on 12% SDS-
polyacrylamide gels followed by staining with Coomassie blue, dry-
ing, 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, Rock-
ford, 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 ac-
cording to the manufacturer’s instruction (Life Technologies). In
each dish (90-mm diameter), 2.5 µg of cDNA of pcDNA3-HA-JNK
and 2.5 µg of cDNA of pEBG-SEK1 wt or pEBG-SEK1 KR mutant
were cotransfected with 1.0 µg 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
Selective Activation of the JNK Pathway during HNE-Induced Apoptosis

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 μM HNE for 6, 16, and 24 h of incubation, respectively, whereas less than 10% of PC12 cells died at 5 and 10 μM 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 μM 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 μM 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 μM HNE, whereas it was maximally (6.8-fold) activated by 10 and 100 μM 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 μM 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).
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 μM 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 activity (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.

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...
appears to affect the JNK activity (lane 4). Pretreatment with the solvent control (lane 1). CPT-cAMP alone did not induce JNK activation. Consistent with our previous results from HNE-induced apoptosis as well as to inhibit the HNE-mediated JNK activation and apoptosis. A, PC12 cells were pretreated with 0.5 mM 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 μM nnonaldehyde, 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 μM nnonaldehyde, 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 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 μM nnonaldehyde, which did not cause apoptosis of PC12 cells (Kruman et al., 1997), did not affect the activity of JNK (lanes 7 and 8).

**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 μM 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 DNA (Esterbauer et al., 1991; Martelli et al., 1994). Under extreme conditions of stress, concentration of newly synthesized HNE may reach up to 100 μM (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...

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 M KK4) and M KK3 (M KK6), an upstream protein kinase of p38 MAP kinase (Rainegaud 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-α, interleukin-1, UV and x-rays, toxic chemicals, and H2O2 (Rainegaud 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 immuno blot data (data not shown but provided to the reviewers). Furthermore, both SEK1 and M KK3 (M KK6 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 (Rainegaud 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 (Rainegaud 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 CCl4 (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-α (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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