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Apoptosis of PC12 cells by 4-hydroxy-2-nonenal is mediated through selective activation of the c-Jun N-Terminal protein kinase pathway

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Abstract

Cytotoxic lipid peroxides such as 4-hydroxy-2-nonenal (HNE) are produced when cells are exposed to toxic chemicals. However, the mechanism by which HNE induces cell death has been poorly understood. In this study, we investigated the molecular mechanism of HNE-induced apoptosis in PC12 cells by measuring the activities of the mitogen-activated protein (MAP) kinases involved in early signal transduction pathways. Within 15–30 min after HNE treatment, c-Jun N-terminal protein kinase (JNK) was maximally activated, before returning to control level after 1 h post-treatment. In contrast, activities of extracellular signal regulated kinase (ERK) and p38 MAP kinase remained unchanged from their basal levels. SEK1, an upstream kinase of JNK, was also activated (phosphorylated) within 5 min after HNE treatment and remained activated for up to 60 min. Marked activation of the JNK pathway through SEK1 was demonstrated by the transient transfection of cDNA for wild type SEK1 and JNK into COS-7 cells. Furthermore, significant reductions in JNK activation and HNE-induced cell death were observed when the dominant negative mutant of SEK1 was co-transfected with JNK. Pretreatment of PC12 cells with a survival promoting agent, 8-(4-chlorophenylthio)-cAMP, prevented both the HNE-induced JNK activation and apop

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tosis. Nonaldehyde, a nontoxic aldehyde, caused neither 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 HNE-mediated apoptosis of PC12 cells is likely to be mediated through the selective activation of the SEK1-JNK pathway without activation of ERK or p38 MAP kinase. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; 4-Hydroxy-2-nonenal; c-Jun protein kinase; Lipid aldehyde

1. Introduction

Increased production of lipid hydroperoxides and peroxides is often observed under pathological or other stressful conditions. Subsequently, highly reactive lipid aldehydes such as 4-hydroxy-2-nonenal (HNE), hydroxyhexanal and malondialdehyde are produced via enzymatic and non-enzymatic reactions [1]. Newly synthesized lipid aldehydes can readily move across cell membranes and can interrupt the normal functions of proteins, DNA and certain organelles [1–3]. Therefore, these lipid aldehydes can directly cause apoptosis of various cells and tissues [1,3–5]. Despite extensive studies on the production of lipid peroxides under stressful conditions, the signaling mechanisms by which lipid peroxides cause cell death have been poorly understood.

Mitogen-activated protein (MAP) kinases are involved in early signaling mechanisms when cells are exposed to various stimuli including growth factors or toxic substances [6,7]. Extracellular signal-regulated kinase (ERK) plays a major role in cell proliferation, differentiation and survival mediated by various growth factors [6]. On the other hand, various inflammatory cytokines and environmental stressors activate the stress-activated protein kinase (SAPK) (also known as c-Jun N-terminal protein kinase, JNK) [8] and p38 mitogen-activated protein kinase (p38 MAP kinase) [9] often in parallel [10]. JNK was recently shown to play an essential role in apoptosis [7], differentiation [11], or oncogenesis [12], depending on the microenvironment of the target cells. It is well established that JNK is activated through upstream protein kinases, including stress-activated protein kinase kinase (SEK1, also known as SAPKK1, JNKK1 or MKK4) and MAP kinase kinase kinase 1 (MEKK1) or apoptosis signal-regulating kinase 1 (ASK1) while p38 MAP kinase is activated by MAP kinase kinase 3 (MKK3, MKK6 or SEK2) and ASK1 [6,10,13].

Recent data demonstrated that HNE caused time-dependent apoptosis of PC12 cells and primary cells of neuronal origin [4,5]. However, PC12 cells were insensitive to HNE-structural analogues such as 2-nonenal and nonaldehyde [4]. In general, the early signaling mechanism during HNE-mediated apoptosis has not been investigated in these cells. The current 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 in PC12 cells.

2. Materials and methods

2.1. Materials

Myelin basic protein (MBP) 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 obtained from Dr J.S. Gutkind (National Institutes of Health, Bethesda, MD). The cDNA for glutathione *S*-transferase-activator of transcription factor 2 (GST-ATF2) fusion protein was from Dr. E.J. Choi (Korea University, Seoul, South Korea). HNE was synthesized and purified before use, as described [14].

2.2. Measurement of HNE-induced cytotoxicity

PC12 cells (2×10^4 cells/well) were grown in collagen precoated 96-well microtiter plates for 2 days in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum, 10% horse serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin and 100 U of fungizocin). After incubation with varying concentrations of HNE (diluted in ethanol, 0.05% final concentration) for indicated times, cells were washed twice with 1 × PBS. Cell viability of remaining cells was determined with the CellTiter96 Non-Radioactive Cell Proliferation assay kit (Promega, Madison, WI) using 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2-tetrazolium (MTS). In some cases, cell death rate was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT), as described [14].

2.3. Immunocomplex kinase activity assay

The soluble fractions (200 μg/reaction) of PC12 or COS-7 cells treated with HNE for indicated times were used to determine the activities of JNK1, ERK and p38 MAP kinase after immunoprecipitation using the respective antibody, as described [14,15]. GST-ATF2 fusion protein was used as a protein substrate for JNK1, and MBP for both ERK and p38 MAP kinase. Other reagents or methods not listed here were the same as described [14].

3. Results and discussion

3.1. Cytotoxic effect of HNE on apoptosis of PC12 cells

To determine the direct effect of HNE on PC12 cell death, we examined HNE concentration- and time-dependent cell death of PC12 cells. As shown in Fig. 1, about 30.3, 30.0 and 67.8% of PC12 cells died upon treatment with 25 μM HNE for 6, 16 and 24 h of incubation, respectively, while less than 20% of PC12 cells died

after exposure to 5 and 10 μM HNE under our experimental conditions. The rates of cell death were significantly increased at higher concentrations of HNE. About 75–85% of PC12 cells died after treatment with 50 and 100 μM HNE for 16 and 24 h. To verify the mechanism of cell death, we stained the cells with 4,6-diamidino-2-phenylindole (DAPI), a sensitive dye for apoptosis. Without HNE treatment, the nuclei of control cells showed uniform staining, indicating that cells were healthy and nuclei were intact. In contrast, after 24 h treatment with 25 μM HNE, more than a half of PC12 cells exhibited typical apoptotic characteristics such as nuclear condensation, as determined by DAPI staining (data not shown), confirming that HNE induces apoptosis of PC12 cells.

3.2. Dose- and time-dependent selective activation of JNK by HNE

To investigate the signaling 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. Throughout our experiments, we always verified by Coomassie blue staining that there were equivalent levels of substrate proteins and MAP kinases separated by SDS-polyacrylamide gel electrophoresis (data not shown). 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 (data not shown). We chose to use 25 μM HNE for our subsequent experiments because this was within the physiological concentration range [1] and led to maximal activation of JNK. HNE treatment activated JNK 4.8-fold within 15–30 min (Fig. 2A). The level of JNK activity returned to background level after 60 min. However, the low basal activities of ERK and p38 MAP kinase remained unchanged throughout the HNE treatment period. Little activation of p38 MAP kinase by HNE was verified using another substrate, ATF-2 for p38 MAP kinase, in the assay [10]. Under our experimental conditions, JNK itself did not appear to be the direct target of HNE

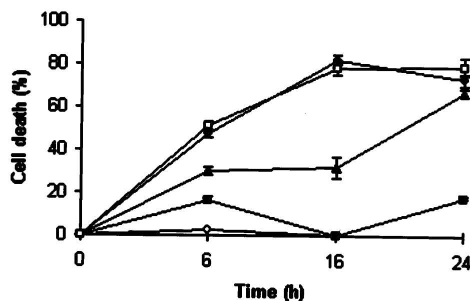


Fig. 1. Time- and HNE-concentration dependent apoptosis of PC12 cells. Time- and HNE-concentration dependent cell death rates were measured by the method described in Section 2: ■, 5 μM HNE; ○, 10 μM HNE; ▲, 25 μM HNE; □, 50 μM HNE; and *, 100 μM HNE. Each point represents the average of five determinants with standard errors. These experiments were repeated at least twice.

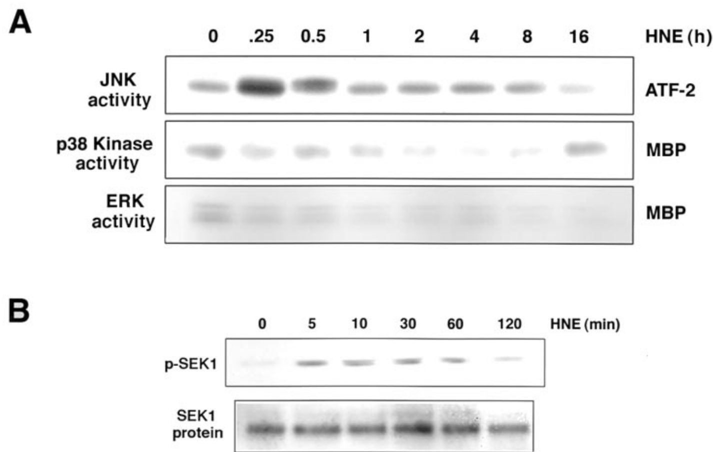


Fig. 2. Time dependent selective activation of JNK and SEK1. (A) HNE-induced changes in the activities of JNK, p38 MAP kinase and ERK in PC12 cell extracts are shown. Each radiolabeled protein was analyzed by SDS-PAGE followed by autoradiography. (B) Time-dependent phosphorylation (activation) of SEK1 by HNE. The soluble fraction (200 μg/well) of PC12 cells treated with HNE for indicated times were subjected to SDS-PAGE followed by immunoblot with a specific antibody against phospho-SEK1 (top) or SEK1 (bottom). The antigen recognized by the primary antibody was visualized by enhanced chemiluminescence.

since HNE did not increase the activity of JNK immobilized on protein G agarose beads (data not shown) or JNK produced in COS-7 cells (see below).

3.3. Phosphorylation of SEK1 by HNE

To verify the activation of the JNK pathway by HNE, the effect of HNE on the phosphorylation (activation) status of SEK1, the immediate upstream kinase of JNK, was analyzed with a specific antibody to phospho-SEK1 or to non-phospho-SEK1. As shown in Fig. 2B (top), the level of phosphorylated SEK1 protein rapidly increased within 5 min after treating PC12 cells with 25 μM HNE, and the activated SEK1 level persisted for about 60 min with little change in the SEK1 protein level (Fig. 2B, bottom) during the same period.

3.4. Activation and role of JNK1-SEK1 pathway in HNE-induced cell death

To confirm the HNE-induced activation of SEK1, we used transient expression of cDNA for HA-JNK, SEK1 wt or dominant negative SEK1 KR mutant in COS-7 cells. HNE treatment did not increase the JNK activity when HA-JNK alone was transfected into COS-7 cells (Fig. 3A, lane 1). Increased JNK activity was observed after HNE treatment (lane 2), when SEK1 wt was co-transfected with

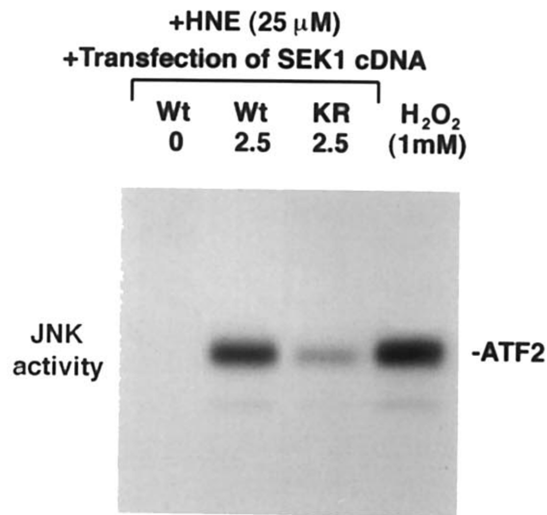
HA-JNK. In contrast, co-transfection of SEK1 KR mutant with HA-JNK markedly reduced HNE-mediated JNK activation (lane 3). Furthermore, H_2O_2 treatment, used as a positive control, increased JNK activity (lane 4), proving that the lack of JNK activation by HNE did not result from the absence of functional JNK in COS-7 cells. These data establish that HNE increases JNK activity through SEK1 activation, although HNE did not seem to directly activate JNK.

To investigate the role of JNK activation in HNE-induced cell death, we determined the relative rates of HNE-induced cell death of COS-7 cells transfected with different combinations of cDNA (Fig. 3B). A small portion (average of 6.6%) of transfected COS-7 cells died in the absence of HNE treatment. After exposure to 25 μM HNE, about 12.2 and 10.0% of cells died after transfection with pcDNA3 vector or HA-JNK plasmid alone, respectively. In contrast, 36.6% of cells died when SEK1 wt was co-transfected with HA-JNK. A significant reduction in cell death was observed with the dominant negative SEK1 KR mutant (14.3%), compared to that with SEK1 wt. These results clearly establish an important role of JNK activation in HNE-induced cell death.

3.5. Effect of CPT-cAMP or nonaldehyde on HNE-induced JNK activation and PC12 cell death

Recent studies suggest that a neurosurvival agent, CPT-cAMP, can promote survival of PC12 cells [16] and other neuronal cells [17] from apoptosis caused by withdrawal of neurotrophic factors. We studied the effect of CPT-cAMP on potential protection of PC12 cells from HNE-induced apoptosis, as well as its ability to inhibit the HNE-induced JNK activation. Consistent with our previous results (Fig. 2A), HNE treatment for 15 and 30 min caused rapid activation of JNK by HNE (Fig. 4A, columns 2 and 3), compared to the solvent control (column 1). CPT-cAMP alone did not appear to affect the JNK activity (column 4). Pretreatment of PC12 cells with CPT-cAMP for 30 min prior to HNE treatment markedly reduced the increase in the HNE-induced JNK activity after 15 and 30 min of HNE treatment (Fig. 4A, columns 5 and 6). Treatment with 25 μM nonaldehyde, which did not cause apoptosis of PC12 cells [4], did not affect the activity of JNK (columns 7 and 8).

Fig. 3. Activation of SEK1-JNK pathway and apoptosis by HNE. (A) Activation of the SEK1-JNK pathway by HNE in COS-7 cells. The respective cDNA constructs (2.5 μg each) for HA-JNK, SEK1 wt and dominant negative SEK1 KR mutant were co-transfected into COS-7 cells (60–70% confluence in 90 mm dishes), using Lipofectamine™ reagent according to the manufacturer's instruction (Life Technologies, Gaithersburg, MD). COS-7 cells were then treated with 25 μM of HNE for 30 min, harvested, homogenized, and immunoprecipitated with HA antibody prior to the measurement of JNK activity. H_2O_2 (1 mM) was used as a positive control. These experiments were repeated at least twice. (B) Effect of transduced SEK1 wt or SEK1 KR mutant on HNE-induced apoptosis of COS-7 cells. COS-7 cells (2×10^5 cells/well) grown in 96-well microtiter plates were transiently transfected with 0.125 μg of expression constructs as indicated, allowed to grow overnight in low serum containing media. After treating with 25 μM HNE for 24 h, cell death rate was determined and presented. * Significantly different ($P < 0.02$) from the corresponding SEK1 wt.



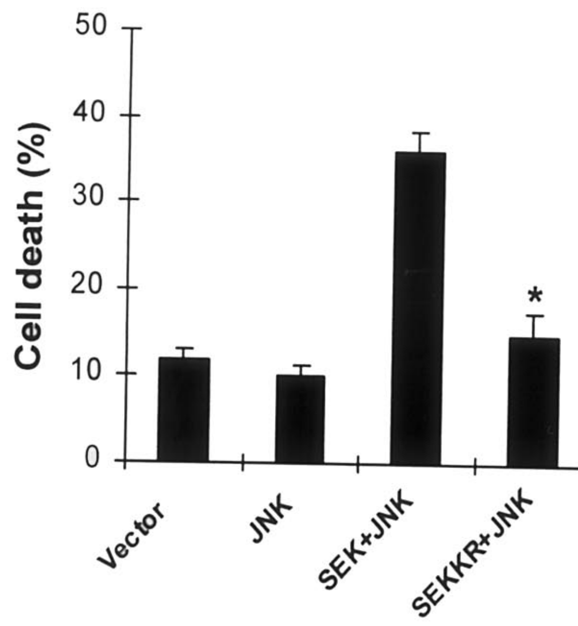


Fig. 3. (*Continued*)

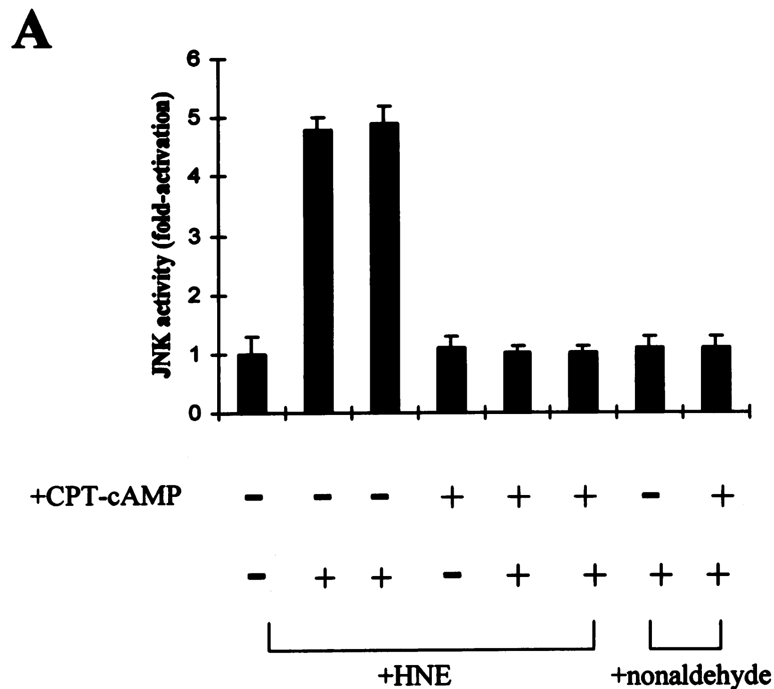
Pretreatment of PC12 cells with CPT-cAMP completely prevented the HNE-induced apoptosis of PC12 cells (Fig. 4B). In addition, 25 μM nonaldehyde, which did not activate JNK (Fig. 4A), did not cause cell death of PC12 cells (Fig. 4B). Taken together, these data indicate that an early JNK activation by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

3.6. Effect of SB203580 on HNE-induced cell death

Horstmann et al. [18] recently reported that specific inhibitors of p38 MAP kinase, SB203580 and SB202190, can 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 pre-treated PC12 cells with SB203580 before HNE-exposure. At two different concentrations of HNE, pretreatment of PC12 cells with either 10 or 50 μM SB203580 did not change the percentage of PC12 cells undergoing apoptosis (data not shown). This result together with our result (Fig. 2A) indicate that p38 MAP kinase is not involved in HNE-mediated apoptosis of PC12 cells.

Despite numerous reports describing HNE-protein adducts, the mechanism by which HNE interferes with cellular functions is poorly understood. To study the molecular process of HNE-induced cell death, we investigated the HNE-mediated changes in the activities of MAP kinases related to cell survival and death. Although Parola et al. [19] recently 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. However, no significant change in the activity of ERK or p38 MAP kinase was observed, even testing under a variety of experimental conditions. The absence of p38 MAP kinase activation by HNE is in contrast with the data by Uchida et al. [20] who recently reported that HNE stimulated both JNK and p38 MAP kinase activity in rat liver epithelial RL34 cells. In fact, p38 MAP kinase and JNK are often up-regulated in a coordinated fashion by various cytotoxic agents such as cytokines including TNF-α, IL-1, UV and X-rays, toxic chemicals and H_2O_2 [10,13,15]. The lack of HNE-induced p38 MAP kinase activation in this study was not due to the absence of this enzyme in PC12 cells, because arsenite can stimulate p38 MAP kinase activity that parallels JNK activity in these cells [21]. In addition, its presence in PC12 cells was detected by our immunoblot analysis (data not shown). Furthermore, osmotic shock, UV irradiation, and an inhibitor of protein synthesis, anisomycin [22], stimulated both SEK1 and MKK3 (MKK6 or SEK2) in PC12 cells, suggesting the presence of a functionally intact p38 MAP kinase-SEK2 pathway in these cells. Thus, our results, taken together with previous results [9,10] suggest that the activation of p38 MAP kinase is dependent on cell type as well as stressors used. The lack of p38 MAP kinase activation by HNE in PC12 cells could result from higher levels or more active PAC1 [10] or MKP-1, which specifically dephosphorylates phospho-p38 MAP kinase [23].

Whether JNK activation or c-Jun phosphorylation is absolutely necessary for the cell death has been actively debated. In some systems such as *c-fos* or *c-Jun* null



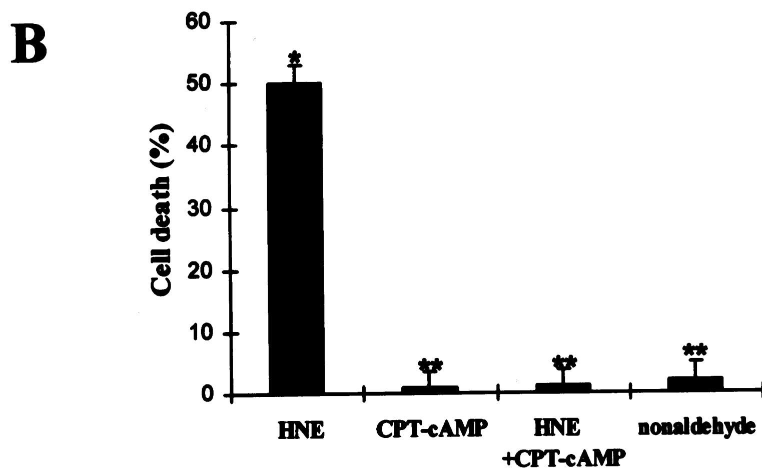


Fig. 4. Effects of CPT-cAMP and nonaldehyde on HNE-induced JNK activation and apoptosis. (A) PC12 cells were treated with 0.5 mM CPT-cAMP for 30 min prior to treatment with 25 μM HNE or nonaldehyde for additional 15 or 30 min. Immediately after HNE or nonaldehyde treatment, PC12 cells were harvested and the activities of JNK in different cell extracts were determined. (B) PC12 cells (2×10^4 cells/well) grown in 96-well microtiter plates for 2 days were treated with 25 μM HNE or nonaldehyde for 16 h in the absence and presence of 0.5 mM CPT-cAMP, as indicated. Cell death rate was then determined and presented. * Significantly different ($P < 0.001$) from the vehicle control. ** Significantly different ($P < 0.001$) from the HNE-treated sample.

embryos [24] or sympathetic motoneurons [25], JNK activation or phosphorylated c-Jun may not be a causal factor in apoptosis. However, for PC12 cells, JNK activation seems to be absolutely necessary since activation of AP-1 activity and over-expression of either c-Jun wt, SEK1 wt or ASK1 wt protein led to cell death [13,26,27] and counteracted the anti-apoptotic effect of bcl-2 [28]. Furthermore, over-expression of the dominant mutant of c-Jun, SEK1 KR, or ASK1 KR prevented apoptotic cell death caused by withdrawal of nerve growth factor [26], dopamine [27], or TNF-α [13]. Additionally, JNK activation appears to be an early event necessary for apoptosis of PC12 cells by ceramide [17], manganese [29] and withdrawal of survival factors [30]. It is also true that motor neuron apoptosis is blocked by a novel inhibitor of the JNK signaling pathway [31]. Therefore, whether JNK activation is a prerequisite for cell death seems to depend on cell type, cell death signal, the duration of JNK activation and the cellular environment such as the presence of another JNK-independent factor, as proposed [15,25].

Since HNE did not appear to directly activate JNK complexed to agarose beads or HA-JNK expressed alone in COS-7 cells (our current data), HNE must act on the upstream kinase of JNK, either SEK1 or its upstream kinase MEKK1 (or ASK1). Although we do not know the mechanism of direct activation of SEK1 or MEKK1 (or ASK1) by HNE, it could be due to activation of MEKK1 (or ASK1) through interaction with various small GTP binding proteins such as Rac or Rho. Alternatively, HNE-mediated activation of SEK1 or its upstream kinase could result from the inhibition of a specific phosphatase which selectively dephosphorylates the phospho-MEKK1 (or -ASK1) or phospho-SEK1.

In summary, the present data demonstrate that HNE selectively activates SEK1 and JNK in PC12 cells with little changes in p38 MAP kinase or ERK. Our data also suggest that JNK activation is critical for HNE-mediated apoptosis of PC12 cells.

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